EVALUATING IGY RECOVERY IN RUMEN FLUID AND ITS POTENTIAL
ROLE IN PERFORMANCE AND NEUTROPHIL FUNCTION IN
LACTATING HOLSTEIN COWS

by

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EVALUATING IGY RECOVERY IN RUMEN FLUID AND ASSESSING ITS POTENTIAL ROLE IN PERFORMANCE AND NEUTROPHIL FUNCTION IN LACTATING HOLSTEIN COWS

by

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ABSTRACT

A series of experiments were conducted to evaluate the effects of a spray dried egg (SDE) product from chickens vaccinated against mammalian enteric pathogens on in vitro disappearance of immunoglobulin Y (IgY) from rumen fluid and on performance of cows fed the product. In two separate *in vitro* experiments we incubated rumen fluid with SDE, an extract of SDE, or starch-encapsulated SDE for different amounts of time and used ELISA to measure IgY disappearance. The objectives of Experiment 1 were to determine IgY recovery from a SDE extract and SDE encapsulated with 50% starch following 6 h of incubation in rumen fluid. We also wanted to determine if recovery was influenced by the addition of total mixed ration (TMR) to the in vitro system. Recovery of IgY from the extract was 26% at 6 h, however, with the addition of TMR, 52% of IgY was recovered at 6 h. Recovery of IgY from starch-encapsulated SDE did not differ from recovery of IgY from the extract. Experiment 2 was conducted to determine IgY recovery from SDE incubated for up to 24 h in rumen fluid with TMR. Recovery of IgY decreased from 47% at 6 h to 25% at 12 h with numeric decreases to 15% and 14% at 18 h and 24 h, respectively. Based on this recovery data we predicted that approximately 50% of IgY from SDE should escape digestion in the rumen and be available to bind with intestinal pathogens.

We elected to evaluate the efficacy of this product on performance and neutrophil function of dairy cows. Thirty lactating Holstein cows (6 primiparous, 24
multiparous) were randomly assigned to a control diet or a diet supplemented with 45 g/d of SDE according to a crossover design with 6-week periods. Feed intake and milk yield were recorded daily and milk composition was determined weekly. Blood was collected every 3 weeks and neutrophils isolated to assess chemotaxis, phagocytosis and oxidative burst. No significant differences were observed in milk yield. There were trends for interactions between treatment and parity on milk yield \((P = 0.10)\) and milk/DMI \((P = 0.06)\), with SDE numerically decreasing performance in primiparous cows and improving performance in multiparous cows. There were trends for a treatment effect on chemotaxis towards media only \((P = 0.09)\) and an interaction between treatment × week on oxidative burst production in stimulated PMN \((P = 0.10)\). In summary, although our \textit{in vitro} experiments demonstrated that a substantial portion of IgY would likely pass from the rumen to the intestines, no benefits of dietary SDE on performance or neutrophil function were observed in this study.
The Innate and Adaptive Immune Systems

**Physical Barriers**

To aid in the protection against invading pathogens, the body utilizes several layers of defense. Physical barriers provide the first layer of defense and include the skin, mucous membranes and tears (Tizard, 2008). The first important physical barrier is the integrity of the skin, which serves as an effective barrier to invading pathogens. Although infections may occur if the skin is damaged, wound healing occurs rapidly. Normal bacterial populations residing on the skin and antimicrobial properties of sweat also aid in defense (Chaplin, 2010; Tizard, 2013). The epithelial barrier serves as the second important physical barrier and is found within the body lining the respiratory, gastrointestinal and urogenital tracts. Epithelial cells, which make up the barrier, form tight junctions to help keep pathogens out that may have been inhaled or digested. Epithelial cells also produce mucous and various antimicrobial peptides that aid in trapping and destroying bacteria.

**Innate Immunity**

The innate immune system relies on the rapid response of chemical and cellular defense mechanisms at the site of infection (Tizard, 2008). Leukocytes, an essential part of innate immunity, are cells called upon to respond to these invading
pathogens and include monocytes, macrophages, dendritic cells, mast cells, neutrophils (PMN), eosinophils, basophils and natural killer (NK) cells (Parkin and Bryony, 2001). Innate immunity relies on the recognition of pathogen associated molecular patterns (PAMPs), evolutionary conserved structures located on the cell surface or within the cell (Akira et al., 2006). Leukocytes, specifically antigen presenting cells such as macrophages, dendritic cells, and B-lymphocytes must be able to recognize PAMPs and are able to do this through pattern recognition receptors (PRRs), which are important in initiating innate and adaptive immune responses (Akira et al., 2006). There are various families of PRRs including C-type lectin receptors (CLRs), NOD-like receptors, RIG-like receptors, and toll-like receptors (TLRs), which have been studied extensively (Medzhitov and Janeway, 2000).

Toll-like receptors are type 1 transmembrane proteins containing leucine rich repeats and a conserved region called Toll/IL-1 (TIR) receptor domain (Werling et al., 2003; Jungi et al., 2011). Ten TLRs have been identified in mammals each having different ligand specificities and they can be divided into two families. Toll-like receptors 1, 2, 4, 5, 6, and 10 are expressed on the cell surface and recognize bacterial, fungal or protozoan products (Iwasaki and Medzhitov, 2004). Toll-like receptors 3, 7, 8 and 9 are expressed internally within endosomal compartments and recognize nucleic acids with discrimination between self and non-self (Iwasaki and Medzhitov, 2004). Toll-like receptor activation via binding to various PAMPs causes different signaling cascades resulting in the production of pro-inflammatory cytokines including interleukin-(IL) 6, IL-8, IL-1β and TNF-α, which can function in recruiting phagocytes to areas of pathogen invasion (Iwasaki and Medzhitov, 2004). Not only are there receptors for pathogenic organisms, microbe-associated molecular patterns
(MAMPs) have been identified as well that are present on the surface commensal bacteria. These MAMPs recognize peptidoglycan, lipopolysaccharide, lipoteichoic acid and single and double stranded DNA unique to prokayotes (Baumgart and Carding, 2007).

**Neutrophil Function**

Neutrophils play a critical part in the innate immune response and serve as the first line of defense against infection. In bovines neutrophils only constitute approximately 20% to 30% of blood leukocytes but are the predominant cell type found in the mammary gland constituting greater than 90% of total leukocytes during early inflammation (Paape et. al, 1981; Sordillo et. al, 1997; Tizard, 2008). Neutrophil function is impaired during the transition period leading to increased risk of cows developing mastitis and metritis (Cai et. al, 1994). The bovine PMN life cycle is short requiring approximately 10-14 days to mature within the bone marrow (Paape et. al, 2002). Once maturation is complete, PMN leave the bone marrow, migrate through endothelia and enter circulation as the circulating pool. Circulating PMN have a half-life of approximately 8 hours within the bloodstream and thus circulate for a short time before entering tissues via diapedesis where they function as phagocytes and perform effector functions or undergo apoptosis (Paape et. al, 2002). A second pool of PMN, known as the marginating pool, remain in capillaries and roll along the vascular endothelium surveying the environment (Parkin and Bryony, 2001; Tizard, 2008).

As PMN are confined in circulation, recruitment to tissues is a multistep process. Inflammatory mediators such as IL-8, LPS and complement component 5a (C5a) stimulate endothelial cells to express glycoproteins, P-selectin and E-selectin, on the cell surface (Paape et. al, 2002; Kobayashi et. al, 2005). P and E-selectins on
endothelial cells cause tethering of PMN to the endothelium followed by rolling along the vessel wall, all mediated by L-selectin. Platelet-activating factor, a lipid secreted by endothelial cells activates rolling PMN resulting in the expression of integrin CD11a/CD18 on the PMN surface, which strongly binds to endothelial intercellular adhesion molecule-1 (Paape et al., 2002; Rambeaud and Pighetti, 2005; Tizard, 2008). This strong binding causes neutrophils to stop movement and firmly attach to the vessel wall despite blood flow. Neutrophils must then migrate through the endothelial barrier in a process called diapedesis, which can occur either paracellurally, between endothelial cells, or transcellularly, through the endothelial cells proper (Hentzen et. al, 2000; Kviets and Sandig, 2001). Once PMN diapedese out of the vasculature and into tissues, they need to be able to recognize inflammatory mediators. Recognition of chemokines occurs through CXC receptors on the surface of PMN. CXCR1 and CXCR2 are two vital chemokine receptors that are required for maximal PMN function during infection (Rambeaud and Pighetti, 2005). Both CXCR1 and CXCR2 can bind to IL-8. CXCR2 can also bind to neutrophil-activating peptide 2 as well as growth related oncogens alpha, beta and gamma (Rambeaud and Pighetti, 2005).

Neutrophils continue to migrate in tissues towards sites of infection in a process known as chemotaxis via the movement towards chemoattractants. Once the target destination is reached, PMN recognize the presence of antigens and antigenic products through the expression of TLRs. Neutrophils can then act as phagocytes and engulf bacteria in a process known as phagocytosis. The pseudopod, containing actin and myosin within the cytoplasm, extends, engulfs and binds with the encountered antigen bringing the antigen into the cell. Various receptors are required for PMN-antigen binding to occur. As the antigen enters the cell it becomes enclosed in a
membrane-bound vacuole called a phagosome (Tizard, 2008; Nordenfelt and Tapper, 2011). Immediately upon binding with bacteria, PMN increase their oxygen consumption significantly by 50-100 fold, which, in turn, activates the NADPH oxidase enzyme complex normally dormant in resting cells (Babior, 2011). The increase in oxygen consumption results in the production of superoxide anion (O$_2^-$) and ROS (Rinaldi et. al, 2007). Reactive oxygen species are important in bacterial destruction, however, they can also cause damage to local tissues resulting in inflammation.

Adaptive Immunity

The innate system is in constant communication with cells of the adaptive system to aid in the elimination of invading pathogens and initiation of immunological memory over time. Similar to the innate system, leukocytes, specifically lymphocytes, carry out adaptive immune responses (Alberts et. al, 2002). Two different classes of lymphocytes, B cells and T cells, are an essential feature to the adaptive immune system. B and T cells develop from similar pluripotent hematopoietic stem cells from which all blood cells develop (Alberts et. al, 2002). In primates and rodents, B cells develop in the bone marrow, whereas, in ruminants and pigs, B cell development occurs in intestinal lymphoid tissues. In all mammals, T cells develop in the thymus (Yasuda et. al, 2006; Tizard, 2008). After development, B and T cells migrate to secondary lymphoid tissues where they encounter antigen.

When B and T cells encounter antigen from antigen presenting cells, such as dendritic cells or macrophages, they proliferate and mature into effector cells capable of eliminating pathogens. Mature B cells, called plasma cells, are able to produce immunoglobulins that are distributed within the bloodstream to target and neutralize
antigens (Alberts et. al, 2002). T cells, on the other hand, do not produce antibodies and are divided into two classes based on cell surface expression (cluster of differentiation; CD): cytotoxic T cells and helper T cells. Cytotoxic T cells (CD8+) kill target antigens through proteins displayed on their surface or by secreting proteins that act locally. Helper T cells (CD4+) work by secreting cytokines that help activate macrophages, B cells, and CD8+ cells (Sordillo et. al, 1997; Alberts, et. al, 2002). It can take days to weeks to develop an adaptive immune response, however, the acquisition of memory can lead to a more rapid and vigorous response upon subsequent antigen exposure. The cell-cell cross-talk between the innate and adaptive immune systems is necessary for effectively eliminating invading pathogens.

**Immunoglobulin Overview: Structure, Function and Antigen Recognition**

Immunoglobulins (Igs) are vitally important for the immune system to identity and eliminate pathogens. Immunoglobulins belong to the immunoglobulin superfamily and consist of two identical heavy (H) and two identical light (L) chains in which the L chain consists of κ or λ chains (Williams and Barclay, 1988). Each H and L chain contains one single amino terminal variable (V) and one or more terminal constant (C) domains. Heavy chains contain three or four C domains while light chains contain only one. Heavy chains that contain three C domains include a spacer hinge region located between the first (CH1) and second (CH2) domains (Williams and Barclay, 1988).

Interactions between Igs and antigens take place between the paratope, the site on the immunoglobulin where antigen binds, and the epitope which is the site on the antigen that the Ig binds to (Paul, 2013). It is important to note that Igs identify surface epitopes on antigens that allows discrimination between two closely related antigens.
This allows for cross-reactivity to occur in which the same Ig can bind divergent antigens that share similar epitopes (Paul, 2013). It has been shown through immunization of different species with monoclonal antibodies that Igs contain both common and individual determinants (Paul, 2013). Common determinants, designated as isotypes, contain epitopes that are specific for the constant portion of the antibody which has allowed grouping of Igs into recognized classes (IgA, IgD, IgE, IgG, IgM in mammals), whereas, individual determinants, called idiotypes, contain epitopes that recognize the V portion of the antibody (Paul, 2013).

Early Ig structure studies used enzymes, papain and pepsin, to fragment IgG molecules into two types of fragments, the antigen binding fragment (Fab) and the crystallizable fragment (Fc) (Paul, 2013; Schroeder and Cavacini, 2010). Papain splits IgG into two Fab fragments that can bind antigen and a single Fc fragment while pepsin splits IgG into an Fc fragment and a single dimeric F(ab)_2 that can cross-link and bind antigens (Paul, 2013; Schroeder and Cavacini, 2010). The Fab fragment is a heterodimer containing a complete L chain and a portion of one H chain, CH1 and is important in that it not only contains the binding site for antigen but allows Igs to discriminate between antigens (Paul, 2013; Schroeder and Cavacini, 2010). On the other hand, the Fc fragment has vital effector functions associated with the humoral immune response and interacts with complement proteins and Fc receptors (FcRs) on immune cells such as mast cells, macrophages and neutrophils (Paul, 2013; Schroeder and Cavacini, 2010). Fc receptors serve as a bridge between humoral and cellular immunity and the action that results from the binding of an Ig to its receptor depends on the receptor, the type of cell it is expressed on and any secondary signals (Paul, 2013; Schroeder and Cavacini, 2010). Several FcRs have been identified which show
specificity for one or two of the Ig isotypes. For example, FcγR is specific for IgG while FcαR shows specificity for IgA (Schroeder and Cavacini, 2010).

**Passive Immunity**

The innate and adaptive immune systems within young animals are functional but not fully developed; therefore, young animals receive maternal antibodies that provide immediate protection from infection until the immune response matures. Passive immunity provides protection that can last several weeks up to three or four months and is essential for survival (Kovas-Nolan and Mine, 2012; Baxter, 2007). In humans, maternal antibodies, mainly immunoglobulin G (IgG), are capable of moving across the placental barrier to the fetus. Within the first six to twelve months of age serum concentrations of maternal IgG begin to diminish. While the infant begins making its own IgG, secretory IgA from breast milk provides protection to the infant by binding to pathogens in the intestinal lumen and, subsequently, inhibiting attachment to mucosal surfaces (Jackson and Nazar, 2006). However, in ruminants and other domestic large animals, placental transfer of antibodies does not occur. Due to the syndesmochorial placenta in cows the maternal and fetal blood supplies remain separate preventing maternal transfer of immunoglobulins (Arthur, 1996). As a result, calves are born agammaglobulinemic requiring ingestion and absorption of immunoglobulins, primarily IgG, from colostrum (Weaver et. al, 2000).

Similar to mammals, birds also require passive transfer of antibodies, however, since the development of the chick occurs outside of the hen, antibody transfer occurs during formation of the egg. Serum IgY is translocated into the yolk through receptor mediated processes on the yolk membrane (Mohammed et al. 1998; Morrison et al. 2001). The hinge region and Fc fragment has been shown to be necessary for this
translocation to occur and the amount transferred is related to the concentration of IgY in serum. It takes approximately five days for the transovarial passage of IgY from serum to yolk to occur. On the other hand, IgA and IgM are deposited into the albumen (Mohammed et al. 1998). During incubation, the developing chick absorbs IgY from the yolk and is able to ingest IgM and IgA from the albumen (Tizard, 2008; Cook and Trott, 2010). Immunoglobulin Y is important in providing passive immunity to various antigens, while IgM functions in agglutination and precipitation of antigens and IgA aids in protecting mucosal surfaces (Tizard, 2008).

**Major Immunoglobulins**

*Immunoglobulin G*

Immunoglobulin G is the predominant isotype found within the blood and extracellular fluid. The concentration of serum IgG in bovines ranges from 1,700-2,700 mg/dl and varies depending on stage of lactation and whether infection is present (Sordillo et al., 1997; Tizard, 2008). Immunoglobulin G has the longest serum half-life compared to other immunoglobulins, and, therefore, plays a vital role in antibody-mediated defense mechanisms (Tizard, 2008; Schroeder and Cavacini, 2010). Taking on a monomeric form, it is the smallest of the immunoglobulins with a molecular weight of 150 kDa and can easily diffuse out of blood vessels into tissues. Immunoglobulin G consists of two identical γ heavy chains and two identical κ or λ light chains (Schroeder and Cavacini, 2010). This Ig plays a direct role in the immune response and functions to neutralize antigens for engulfment by phagocytes (Janeway et. al, 2001). Various subclasses of IgG exist, each having different binding affinities for FcγR and biological functions depending on the species (Park et. al, 1992; Tizard,
The major subclasses in bovines include IgG₁ and IgG₂. Both can function to augment phagocyte function of neutrophils and macrophages (Sordillo et al, 1997).

**Immunoglobulin M**

Immunoglobulin M, similar to IgG, is produced in the bone marrow, lymph nodes and spleen by plasma cells. It accounts as the second highest concentration in most mammalian serum after IgG (Tizard, 2008) ranging between 250-400 mg/dl in bovines. Immunoglobulin M exists as a monomer of 180-kDa expressed on the surface of naïve B cells. The monomer contains two light chains, either κ or λ, and two μ heavy chains (Schroeder and Cavacini, 2010). As B cells mature upon antigen stimulation, pentameric IgM is secreted in which monomers are linked together by disulfide bonds (Janeway et al, 2001; Tizard, 2008). The pentamer also contains a polypeptide chain, known as the J chain, which aids in secretion at mucosal surfaces. Like IgG, IgM also neutralizes antigens, activates complement and plays a role in immunoregulation (Boes, 2000; Schroeder and Cavacini, 2010).

**Immunoglobulin A**

While IgG is the predominant isotype found in blood, Ig A is the predominant isotype found at mucosal surfaces such as in the respiratory, urinary and intestinal tracts as well as the mammary gland and skin. In most mammals the serum concentration of IgA tends to be lower than that of IgM accounting only for 10-50 mg/dl in bovines (Tizard, 2008). In serum, IgA is expressed on the surface of plasma cells as a monomer weighing 150 kDa and consists of two light chains and two α heavy chains. In mucosal secretions, however, IgA, called secretory IgA, exists in the
form of a dimer containing a J chain and a secretory component (Schroeder and Cavacini, 2010). Secretory IgA (S-IgA) is essential in protecting mucosal surfaces from bacteria, viruses and toxins through neutralization or by preventing adherence to the mucosal surface (Schroeder and Cavacini, 2010; Tizard, 2008; Janeway et. al, 2001).

**Immunoglobulin Y**

Immunoglobulin Y is the major antibody found in avians and has similar functions as mammalian IgG. Differences between IgY and IgG are apparent in structural composition. Comparable to mammalian IgG, avian IgY is the predominant serum Ig and exists as a monomer. Avian IgY is also composed of two identical heavy and two identical light chains linked by disulfide bonds forming a Y shape (Murai, 2013). Structural differences between avian IgY and mammalian IgG arise within the heavy chain. The heavy chain of avian IgY contains an additional constant domain resulting in a higher molecular mass of 180 kda compared to the molecular mass of 150 kda in mammalian IgG. Another structural difference is observed in the number and location of N-glycosylation sites between avian IgY and mammalian IgG (Murai, 2013; Warr et. al, 1995).

**Mucosal Immunity of the Gastrointestinal (GI) Tract**

**Structure of the GI Mucosa**

The gastrointestinal tract is a long, complex tube that begins at the mouth and extends to the anus. While the mouth and stomach are important in breaking down and digesting feed, the functionality of the small and large intestines within the lower GI tract is two fold: 1) to further digest feed for nutrient absorption and 2) to aid in innate
and adaptive immune responses to potential pathogens while maintaining immunologic tolerance towards the host’s commensal microbiome. This dual function occurs in the mucosa, which surrounds the lumen or inner part of the tube.

A single epithelial cell layer, covered by mucus, protects the mucosa by acting as a barrier to prevent pathogens from adhering and penetrating through the epithelial layer potentially causing infection. Several different intestinal epithelial cell (IEC) types make up the epithelial layer and include enterocytes, goblet cells, Paneth cells, and microfold (M) cells, each having unique functions. Enterocytes, also called columnar epithelial cells, mainly function to absorb nutrients but also protect the mucosa by firmly attaching to adjacent enterocytes via tight junctions to form a surface barrier. Goblet cells produce mucus and antimicrobial peptides, regulated by TLRs, that function to prevent bacteria from directly attaching to the epithelial layer (Sodhi et al., 2012; Johansson et al., 2013). Mucus layers are organized differently within the small and large intestines. A single layer of mucus, which is not attached to the epithelium, is present in the small intestine. Two layers of mucus, an inner and outer layer, exist in the large intestine (Atuma et al., 2001 and Ermund et al., 2013). The inner layer is firmly attached to the epithelium and is continuously renewed by goblet cell secretion. Paneth cells secrete antimicrobial peptides, such as RegIII-γ and RELM-β, which aid in innate immune defense by preventing pathogens from adhering to the innermost layer of mucus. M-cells are specialized cells that differentiate from epithelial cells in response to stimulation from B-cells that express LTα1β2 (Mowat, 2003; Mason, 2008). These cells function in initiating or suppressing acquired antigen specific immune responses via the uptake and transport of antigens from the intestinal
lumen across the epithelial barrier to antigen presenting cells, such as dendritic cells (Neutra, 1999; Mason, 2008; Kurashima et al., 2013).

**Gut Associated Lymphoid Tissue**

The gut associated lymphoid tissue (GALT) is important in determining the type of immune response to be elicited; whether tolerogenic to maintain the host’s microbiota or adaptive to eliminate pathogens. Gut-associated lymphoid tissue can be divided into effector and inductive sites. Effector sites include large numbers of lymphoid cells dispersed throughout the epithelium and lamina propria (LP). The LP lies beneath the mucosal epithelium and contains DCs, macrophages and lymphocytes important in antigen presentation to CD4+ T cells. The LP is also important in B cell expansion and differentiation into plasma cells (Brandtzaeg and Johansen, 2005).

Intraepithelial lymphocytes, a population of T cells interspersed through the epithelial layer, function to maintain the epithelial barrier as well as eliminate pathogens via the production of various inflammatory mediators such as TNF-α and IFN-γ. Innate lymphoid cells, which lack adaptive B and T cell properties, play an important role in regulating intestinal homeostasis. Immune responses have been designed to induce tolerance to the commensal intestinal microbiome but, at the same time, must be able to identify and respond to potentially pathogenic organisms (Mason, 2008; Taschuk and Griebel, 2012). Inductive sites include mesenteric lymph nodes, Peyer’s patches (PP), cryptopatches and isolated lymphoid follicles. Peyer’s patches are lymphoid aggregates found throughout the small intestine and contain germinal centers that promote antigen-specific T cell and B cell interactions (Mowat, 1997; Mason, 2008). Follicular associated epithelium, an area rich in M cells, covers the PP. Cryptopatches, located within crypts, are small mucosal lymphoid aggregates containing lymphoid
precursors and are scattered throughout the GI tract. Isolated lymphoid follicles are also found in the small and large intestine and contain primarily B cells (Gutzeit et al., 2014).

**Microbial Colonization of the GI Tract**

The intestinal microbiota plays a crucial role in nutrition and health. The microbiota break down dietary carbohydrates, produce short chain fatty acids, and synthesize vitamins. It can also limit mucosal adherence of pathogenic bacteria through competition for nutrients or attachment sites. The gastrointestinal tract becomes colonized by a diverse microbial population shortly after birth. *Bifidobacterium* and *Lactobacillus* are the primary bacterial species that colonize the newborn human’s intestinal tract as these bacteria are derived from the vaginal canal and breast milk (Cerutti and Rescigno, 2008). Smaller numbers of *Bacteroides*, *Clostridia*, and *Escherichia coli* are also present within the GI tract (Bullen et al. 1976). The shift to solid food with weaning results in a more diversified microbial population more similar to what is seen in the adult gut. While the intestinal tract is inhabited by thousands of species, *Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria* are the predominate phyla. *Bacteroidetes* accounts for approximately 23%, whereas, *Firmicutes* constitutes around 64% of the total microbiota within the GI tract (Sartor and Mazmanian, 2012). The large intestine and colon are dominated by the phyla *Bacteroidetes* and *Firmicutes* (Marteau et al. 2001). Bacterial densities within the duodenum and jejunum of the small intestine are approximately $10^3$–$10^5$ per gram of luminal contents while higher densities can be found in the distal ileum at $10^8$ organisms per gram (Cerutti and Rescigno, 2008). Bacterial densities within the colon
can reach approximately $10^{10} - 10^{12}$ organisms per gram and encompass thousands of species (Cerutti and Rescigno, 2008).

The diverse microbial population is important in the development of GALT, as well as, modulating local and systemic immune responses. Studies conducted in mice have demonstrated that intestinal function and immune response are regulated by the microbiome. Additionally, the microbiota can drive the expansion of lymphocytes, specifically CD4+ T cells, including T-regulatory cells expressing Foxp3 (Unutmaz and Pulendran, 2009). These cells aid in unwarranted inflammatory responses. From germ free studies conducted in mice, it has been shown that commensals have specific roles in the immune response. For instance, colonization of mice with *Clostridia* species results in IL-10 production from T-reg cells (Atarashi and Honda, 2011) and *Bifidobacteria* play a role in the maturation of the s-IgA system (Ivanov et al., 2009). The commensal bacteria are important in maintaining intestinal homeostasis and help drive the immune response towards an anti-inflammatory state.

**Dysbiosis and the Inflammatory Response**

Dysbiosis results when dramatic shifts occur within the microbial population that can lead to alterations in the host’s metabolism and immune responses. Dysbiosis is implicated in the onset of intestinal inflammatory disorders such as colitis, Crohn’s disease, and inflammatory bowel disease (IBD) (Sartor and Mazmanian, 2012). For example, a study conducted by Frank et al. (2007) demonstrated a decrease in *Bacteriodetes* and *Firmicutes* with an increase in *Actinobacteria* and *Proteobacteria* in intestinal samples from patients with IBD. In patients with IBD, commensals such as *E. coli* contribute to inflammation by adhering to the mucosa, and translocating through the epithelial layer. (Baumgart et al., 2007; Darfeuille-Michaud et al., 2004).
In Crohn’s disease, commensals such as *E. coli* and *B. fragilis* species can invade mucosal ulcers and can cause systemic complications such as endocarditis or hepatic lesions (Sartor, 2008; Keighley, 1982). Dysbiosis also accompanies subacute ruminal acidosis (SARA), a common metabolic problem in early lactating dairy cows, caused by the switch to diets containing higher proportions of starch in order to support the high energy demands required of milk production. Starch is highly degradable in the rumen resulting in the production of high amounts of volatile fatty acids that lead to SARA. Khafipour et al. (2009) have shown that high grain diets induce a shift towards a higher proportion of gram-negative bacteria, specifically *Streptococcus bovis* and *E. coli*, in the rumen. In mild-grain induced SARA, a higher proportion of *Megasphaera elsdenii* was observed. The result of SARA is decreased rumen pH and increased LPS concentrations in the rumen and intestines, which can alter intestinal permeability and lead to systemic inflammation (Khafipour et al., 2009).

When potentially pathogenic organisms elicit proinflammatory responses at the mucosal level, various cytokines and chemokines are produced and aid in PMN recruitment (Kucharzik et al., 2005; McCormick et al., 1995). In addition, B cells differentiate into IgA producing plasma cells and coat bacteria. Fc receptors recognize the constant regions of IgA resulting in phagocytes binding to and engulfing IgA coated bacteria. Upon coating of IgA, bacteria can also be neutralized (Janeway et al., 2001). Macrophages and PMN along with epithelial and lymphoid cells are important in the resolution of inflammation and the healing process. In the case of intestinal disorders, increased production of IL-8, IL-17 and LTB4 leads to increased PMN recruitment, which can result in damage to the epithelial layer and increased inflammation. Massive numbers of infiltrating PMN across tight junctions can lead to
the formation of gaps between IECs (Milks et al., 1983; Nash et al., 1987). Gap formation can cause epithelial damage and increased barrier permeability (Nash et al., 1987; Nusrat et al., 1997). Crypt abscesses in the colon can result from accumulation of PMN within epithelial crypts resulting in morphological alterations (Xavier and Podolsky, 2007) (Figure 2). Massive amounts of infiltrating PMN can lead to a decline in barrier integrity. As a result, opportunistic pathogens have the ability to adhere to and translocate through the mucosa, which causes further inflammatory responses. Therapeutic treatments, such as prebiotic and probiotics, have been targeted to correct the dysbiosis seen in patients with intestinal diseases. For instance, patients with Crohn’s disease are deficient in the Firmicutes phylum, particularly in the *Clostridium leptum* group. Oral administration of *F. prausnitzii*, one of the most prevalent bacteria within the gut and a member of the *C. leptum* group, has been shown to reduce inflammation and correct this microbial imbalance (Sokol et al., 2008).

**Systemic Inflammation and Overall Health**

When localized inflammation is not completely resolved, a systemic inflammatory response occurs, known as the acute phase reaction. The decline in epithelial barrier function results in bacteria and bacterial products traveling to the liver and entering the circulation. As a result of tissue injury and infection, increased levels of acute-phase proteins, such as C-reactive protein and $a_1$-acid glycoprotein, have been linked to intestinal diseases in humans including Crohn’s disease and ulcerative colitis (Vermeire et al., 2004). The systemic immune response is in a constant state of activation as evidenced by activation of the complement system (Hodgson et al., 1977). Chronic inflammation in Crohn’s disease and ulcerative colitis has been known to cause other disorders including psoriasis, arthritis and inflammation in the eyes.
(Baumgart and Sandborn, 2007). Similarly, SARA can elicit an acute phase reaction resulting in increased levels of the acute phase proteins, haptoglobin and serum amyloid A. The chronic inflammatory response in cows with SARA results in depressed feed intake, reduce milk production, liver abscesses and lameness (Krause and Oetzel, 2006).

**Circulating PMN Function during Inflammatory Disorders**

Alterations in circulating PMN function are associated with chronic inflammatory disorders. Nikolaus et al. (1998) demonstrated *in vitro* that circulating PMN isolated from patients with IBD or colitis released increased amounts of IL-1β and TNF-α upon LPS stimulation compared to PMN from healthy controls. This suggests an increased level of primed PMN in circulation in individuals with active intestinal disease (Nikolaus et al., 1998). Trottier et al. (2012) observed that superoxide production from unstimulated PMN was 59.6% greater in PMN from obese patients compared to PMN from lean individuals. However, superoxide production in response to activation with 0.01 μmol/l phorbol myristate acetate was reduced by 27.9% (Trottier et al., 2012). This suggests that although circulating PMN in obese individuals may be primed, these cells exhibit moderately impaired function upon an activating stimulus. In response to an *in vitro* LPS challenge at doses of 0.1 ng/ml and 1 ng/ml, PMN apoptosis significantly decreased in obese individuals compared to lean individuals suggesting that obesity alters PMN responsiveness, however, no difference was noted at 10 ng/ml (Trottier et al., 2012). In a separate study by Nijhuis et al. (2009), the researchers found that circulating levels of myeloperoxidase and calprotectin, proteins stored in the cytoplasm or granules and released following PMN activation, were increased in obese individuals compared to
normal weight individuals. Membrane expression of an activation marker, CD66b, was also assessed on circulating PMN and expression was increased in obese individuals compared to lean controls (Nijhuis et al., 2009). This suggests sustained activation of circulating PMNs occurs under persistent inflammation. Alvarez-Rodriguez et al. (2013) observed a significant increase in chemotaxis in unstimulated PMN from patients with early onset rheumatoid arthritis compared to healthy individuals. They also observed that once the disease was treated, no significant changes in PMN chemotaxis were seen (Alvarez-Rodriguez et al., 2013). Taken together, these studies suggest that circulating PMN remain activated and hyper responsive under conditions of inflammation.

**IgA Production, Transport and Function**

Mucosal surfaces, specifically of the GI tract, continuously come into contact with luminal contents. Large amounts of IgA are produced by plasma cells in the GALT and are mainly secreted as dimers (Macpherson et al., 2008). The majority of the IgA responses within the GI tract are initiated within inductive sites, specifically PPs, which produce cytokines, such as transforming growth factor β (TGF-β), that have IgA-inducing functions (Gonnella et al., 1998). Peyer’s patches also produce IL-4, IL-6 and IL-10, which all serve to facilitate the expansion of B cells expressing IgA as well as influence B cell differentiation to IgA-secreting plasma cells (Defrance et al., 1992). Evidence in mice has shown that IgA is also produced by plasma cells in the lamina propria and MLNs (Yamamoto et al., 2004).

Dimeric IgA secreted by plasma cells within PPs and LP must be transported from the basal to the apical side of IECs in order to fulfill its protective role, and it arrives there via transcytosis, a process mediated by the polymeric immunoglobulin
receptor (pIgR) (Mostov, 1994). Expression of pIgR is regulated by pro-inflammatory cytokines such as interferon-γ, IL-1 and TNF-α (Asano and Komiyama, 2011). Within IECs, newly synthesized pIgR travels to the trans-Golgi network where it is then sorted into vesicles and delivered to the basolateral surface where binding of pIgR to IgA occurs (Kaetzel, 2005). The pIgR carries IgA through various compartments within IECs until it reaches the apical surface at which point pIgR is cleaved resulting in release of secretory IgA (Kaetzel, 2005).

Once s-IgA reaches the apical surface, it can prevent bacteria, whether pathogenic or commensal, access to the epithelium, known as immune exclusion. While the s-IgA does not inhibit bacterial growth, s-IgA can prevent commensals from adhering to the epithelial surface by binding to specific epitopes on the bacterial surface (Brandtzaeg et al., 1968; Brandtzaeg, P, 2013). It has been observed that about 40% of anaerobic bacteria in human feces is coated with s-IgA (van der Waaij et al., 1996). However, studies conducted in mice have shown that IgA coating of commensals is unrelated to the total s-IgA excreted to the intestinal lumen. This suggests that innate and specific IgA binding properties may be involved in the coating of commensals (Kamada et al., 2013; Tsuruta et al., 2009). Potentially pathogenic commensals, termed pathobionts, may show increased percentage of bacteria coated with IgA within the lumen. For instance, dysbiosis can result in an increased fraction of IgA coating on anaerobic bacteria, which is observed in patients with IBD (Kamada et al., 2013; Robles Alonso and Guarner, 2013). Coating of commensals by s-IgA contributes to immune homeostasis by attenuating the host’s pro-inflammatory responses by blocking adherence to the intestinal epithelium.
Specific s-IgA is important in controlling pathogenic infections by binding and neutralizing bacteria at the mucosal layer. Through coating, s-IgA is able to dampen the pro-inflammatory responses to pathogenic bacteria. A study was conducted using rabbits as a model of *Shigella flexneri* infection to assess this role of IgA (Boullier et al., 2009). *S. flexneri* coated with s-IgA was injected into ileal tissue and was detected in the subepithelial dome of Peyer’s patches (Boullier et al., 2009). This group observed that s-IgA coated *S. flexneri* resulted in decreased amounts of IL-6, TNF-α, IFN-gamma and maintained a sustained level of IL-10 (Boullier et al., 2009). By reducing pro-inflammatory responses, s-IgA is essential in maintaining the integrity of the epithelial barrier.

**The IgY Advantage**

There has been increasing interest for using chickens over mammals in the production of polyclonal antibodies against a variety of antigens as a source of passive immunity for humans and animals alike (Kovas-Nolan and Mine, 2012). Although both mammals and chickens must be immunized to develop specific antibodies, antibody harvest from mammals is more invasive requiring collection of blood compared to daily egg collection in chickens (Munhoz et. al, 2014). Hens can be used throughout their entire egg laying period for antibody production. Compared to laboratory animals, such as rabbits or mice, chickens produce higher amounts of specific antibodies. Even though the amount of IgY within yolk varies depending on the age and breed of the bird, as well as yolk size and antigen used, the amount of IgY typically ranges from 50 to 100 mg per egg (Schade et al., 2005; Trott et al., 2005; 2009a; Cook and Trott, 2010) with an estimated amount of 2 to 10% being antigen specific (Schade et al., 2005; Cook and Trott, 2010; Table 1).
Higher antibody production may be due in part to stronger immune responses upon immunization. It has been shown that, on average, chickens exhibit a higher avidity, the overall binding strength between an antibody and an antigen, \((10^9 \text{ mol/L})\) compared to the avidity observed in sheep \((10^8 \text{ mol/L})\) after an initial immunization (Wooley and Landon, 1995). Also, small amounts of antigen (0.1-1 \(\mu\)g) are required to elicit an immune response in chickens (Larsson et. al, 1998; Carlander, 2002). Due to a large phylogenetic difference between IgY and IgG, immunological cross-reactivity between the two is non-existent, however, cross-reactivity does occur between IgG from different mammalian species (Kovas-Nolan and Mine, 2012). Unlike IgG, IgY does not activate the complement system and can be used in place of IgG to reduce interference by complement activation (Kovas-Nolan and Mine, 2012).

**Table 1. Characteristics of avian IgY and mammalian IgG**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Avian IgY</th>
<th>Mammalian IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody sampling</td>
<td>Non-invasive</td>
<td>Invasive</td>
</tr>
<tr>
<td>Amount of antibody per harvest</td>
<td>50-100 mg of IgY per egg</td>
<td>200 mg of IgG per bleed</td>
</tr>
<tr>
<td>Amount of antibody per month</td>
<td>1,500 mg</td>
<td>200 mg</td>
</tr>
<tr>
<td>Amount of specific antibody</td>
<td>2-10%</td>
<td>5%</td>
</tr>
<tr>
<td>Interference with IgG</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Activation of mammalian complement</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Adapted from Schade et al., 2005

**Can IgY Enhance Immune Function?**

The use of IgY has been beneficial in both the human and veterinary medical fields as an alternative to antibiotics and other therapeutic remedies. Several studies have been conducted to determine the effectiveness and proposed use of IgY against
various human and animal pathogens within laboratory and clinical settings (Kovas-Nolan and Mine, 2012).

Two important clinical applications of IgY use in humans include the prevention of infections due to \textit{Helicobacter pylori} and \textit{Pseudomonas aeruginosa} (PA) colonization. \textit{H. pylori} is a common cause of gastritis and peptic ulcers and there has been evidence of antibiotic resistant strains. Shin et al. (2002) demonstrated a reduction in bacterial growth and adhesion in an \textit{in vitro} model and reduced gastric inflammation in an animal model. In their study, Mongolian gerbils were administered a daily oral dose of either 1 mg or 10 mg of IgY against \textit{H. pylori}. Mucosal injury was reduced in gerbils who received the higher dose due to decreased numbers of adhered \textit{H. pylori} resulting in lower numbers of lymphocyte and neutrophil infiltrates. However, cross-reactivity may occur within the human microbiome if IgY is produced against whole cell \textit{H. pylori}. To avoid this potential cross reaction, IgY produced against urease, which is important in \textit{H. pylori} metabolism and virulence, has been shown to be beneficial (Mobley, H.L.T. 2001; Shin et al., 2002). In a study conducted by Horie et al. (2004), volunteers positive for \textit{H. pylori} were given a yogurt containing \textit{Lactobacillus acidophilus} and \textit{Bifidobacterium spp.} and supplemented with 1.5 g of egg yolk IgY produced against urease three times daily (45 mg of IgY-urease/d), and, within 4 weeks, there was a significant reduction in urea and antigen detection in feces. \textit{Pseudomonas aeruginosa} causes the majority of morbidity and mortality in cystic fibrosis patients through colonization of the airways. It is difficult to treat infections once they have become established, and antibiotic resistant strains have developed. Ongoing trials in CF patients have demonstrated that a mouth rinse containing purified IgY against \textit{P. aeruginosa} (50 mg of IgY) given daily can reduce
or prevent colonization of the respiratory tract (Carlander et al., 2000; Kollberg et al., 2003; Nilsson et al., 2007; 2008).

Immunoglobulin Y has also been suggested for defense against biological warfare agents such as *Staphylococcal aureus* enterotoxin B (SEB) and *Clostridium botulinum* neurotoxins. LeClaire et al. (2002) injected mice with a mixture containing 200 ug of IgY antibodies to SEB or rhesus monkeys with 10 mg/kg of IgY antibodies to SEB. Animals that received the anti-SEB IgY 20 min before or 4 h after challenge were protected upon a lethal SEB challenge. In another study, Pauly et al. (2009) found that the activity of *C. botulinum* neurotoxins was blocked by use of purified IgY in vitro and demonstrated the same blocking effect in mice when the toxin was preincubated in toxin-specific IgY.

In production animals, several studies have been conducted in swine looking at the effects of IgY on performance and overall health. In a study conducted by Hong et al. (2004), 36 pigs were assigned to a diet supplemented with either 0.08% of an antibiotic, 0.1% of sprayed dried egg yolk antibodies (SDEP) or 0.2% of SDEP specific against *E. coli*. This group observed that pigs fed the 0.2% SDEP had numerically higher weight gains and were more feed efficient compared to pigs fed the diet containing the antibiotic (Hong et al., 2004). Song et al. (2012) also observed an increase in weight gain when feeding diets supplemented with 5% spray dried egg from unvaccinated hens to weaned pigs. The improvement in performance of non-challenged pigs in response to dietary IgY was proposed to be due to reductions in immune activation, sparing energy which in turn was used for growth. Owusu-Asiedu et al. (2003) looked at performance in pigs challenged with the K88 strain of *E. coli* then either fed a control diet (no supplementation) or a diet supplemented with 0.5%
egg yolk antibodies from hens immunized with enterotoxigenic *Escherichia coli* (ETEC) or a diet supplemented with the antibiotic, Carbadox. Weight gain in pigs fed the diet containing IgY (151.2 g/d) was comparable to pigs fed the diet containing Carbadox (152.6 g/d) with higher weight gain in both supplemented diets compared to the control (100.9 g/d) (Owusu-Asiedu et al., 2003). In another study looking at performance and diarrhea incidence in weaned pigs orally dosed with 40 mg IgY (non-encapsulated or microencapsulated) from hens vaccinated with ETEC K88 then challenged with *E. coli* (Table 2), Li et al. (2009) observed that pigs who received either form of IgY had improved weight gain and recovery rates compared to pigs who received aureomycin. This suggests that IgY is more beneficial in improving performance and immune health compared to antibiotics.

Table 2. Effect of encapsulation of IgY on performance and the incidence of diarrhea in pigs challenged with *E. coli*

<table>
<thead>
<tr>
<th></th>
<th>Percentage of pigs with diarrhea after specific times (Fecal score in parentheses)¹</th>
<th>Weight gain (g/d)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Negative control</td>
<td>0%  (0.5)</td>
<td>0% (0.0)</td>
</tr>
<tr>
<td>Positive control</td>
<td>75% (2.5)</td>
<td>75% (2.5)</td>
</tr>
<tr>
<td>Non-encapsulated IgY</td>
<td>100% (2.0)</td>
<td>75% (1.3)</td>
</tr>
<tr>
<td>Microencapsulated IgY</td>
<td>75% (2.0)</td>
<td>0% (0.0)</td>
</tr>
<tr>
<td>Aureomycin</td>
<td>100% (2.0)</td>
<td>50% (2.0)</td>
</tr>
</tbody>
</table>

¹Fecal score is the mean fecal consistency score where 0 = normal, 1 = soft feces, 2 = mild diarrhea, 3 = severe diarrhea.
²Gain over a 72 h period.
Studies conducted in bovines have shown some promising results. Maintaining optimal rumen pH is key for the prevention of rumen acidosis and associated health disorders. A study conducted by Di Lorenzo et al. (2006) looked at the effects of feeding IgY against *Streptococcus bovis* and *Fusobacterium necrophorum* to ruminally cannulated steers fed a high-grain diet on ruminal bacterial populations and pH. Feeding IgY reduced bacterial population counts of *S. bovis* and *F. necrophorum* and increased pH compared to the control (Di Lorenzo et al., 2006). DiLorenzo et al. (2007) conducted a similar study in ruminally cannulated Holstein cows fed IgY not only against *S. bovis* and *F. necrophorum*, but also against *Clostridium stricklandii*, *Clostridium aminophilum*, and *E. coli* O157:H7 and found that rumen pH increased and lactate concentration decreased in response to IgY.

Zhen et al. (2008) conducted an *in vitro* study evaluating the activity of IgY from hens immunized with *Staphylococcus aureus*, which is a common cause of mastitis in dairy cows, on bacterial growth and phagocytosis by macrophages isolated from milk. The authors observed that IgY inhibited bacterial growth as well as increased phagocytic activity of milk macrophages. A follow up *in vivo* study was conducted using 18 lactating cows with *S. aureus* clinical mastitis and 18 lactating cows with *S. aureus* experimental mastitis randomly assigned to one of three treatments infused through the teat canal into the lumen of the quarter: no infusion, IgY (200 mg) infusion or penicillin (2,000 mg) infusion. Increased recovery rates were observed in both experimental and clinical mastitis cows assigned to the IgY treatment (83.3% and 50%, respectively) compared to cows assigned to the penicillin infusion (66.7% and 33.3%, respectively) (Zhen et. al, 2009).
Dairy Cow Health and Feed Supplementation

From genetic selection to higher quality forages and housing modifications, milk production in cows has doubled since 1957 (Hansen, 2007; Ingvartsen, and Moyes, 2013). While milk production has improved, unfortunately, the rates of recorded disease incidences have not (Ingvartsen and Moyes, 2013). A variety of factors play a role in disease incidence including nutrition, immune health and management practices regarding disease prevention. In recent years, these factors have received notable attention during the transition period, also known as the periparturient period, of the cow (Drackley, 1999). This period is typically defined as three weeks prior to calving and continuing up until three weeks post calving. During this time, cows experience changes in metabolism due to hormonal and dietary shifts. These changes commonly lead to immunosuppression and systemic inflammation leading to increased incidences of displaced abosmasum, mastitis, metritis, milk fever and fatty liver although some cows are more susceptible than others (Mallard et. al, 1998; Ingvartsen, 2006; Ingvartsen and Moyes, 2013).

Feed additives have been studied in an effort to boost overall health during the transition period and early lactation and some have been proven to be successful. For instance, mineral and vitamin supplementation have been shown to improve immune function. Briefly, selenium and vitamin E, important antioxidants, help control inflammation by contributing to the neutralization of reactive oxygen species (ROS) and studies have shown decreases in rates of clinical mastitis (Smith et al., 1984, Weiss et al., 1990) in response to supplementation. Vitamin A and B-carotene can destroy free radicals and have been shown to improve udder health and decrease rates of metritis and retained placenta (Michal et al., 1994; Sordillo et al., 1997). Zinc,
copper and manganese improve epithelial barrier integrity against infection and are important in various antioxidant pathways (Nemec et al., 2012).

Within recent years, feed supplementation with different formulations of prebiotics and probiotics on animal health has been investigated. It has been demonstrated in monogastric animals that feeding prebiotics and probiotics can enhance the immune response at the intestinal mucosal level and cause shifts in the intestinal microbiome (Brown et al., 2012). While digestion in ruminants is more complex due to pregastric fermentation, feeding prebiotics and probiotics to dairy cows may be beneficial to overall health. For instance, studies have been conducted looking at the effects of live yeast and yeast products, most commonly \textit{Saccharomyces cerevisiae}, on dairy cow health. Yeast is known to function primarily as a prebiotic because both live and killed yeast lead to similar changes in digestion (Oeztuerk et al., 2005; Oeztuerk, 2009). Feeding yeast supplements to dairy cows can improve fiber digestion, increase rumen pH and can alter the rumen microbiome (Chaucheyras-Durand and Durand, 2010; Pinloche et al., 2013).

As shown from the studies above, IgY added to feed modifies bacterial populations within the rumen of dairy cattle and can aid in treating mastitis when infused into the mammary gland. While health benefits have been observed, not much work has been conducted to determine if IgY can survive within the rumen environment and reach the small intestine. If IgY can survive, feeding dairy cattle IgY specific for enteric pathogens may prevent adhesion of pathogens to the epithelial layer which may dampen the immune response thus reducing intestinal inflammation. The reduction in inflammation may increase available energy that may be used to increase milk production. The purpose of this thesis was to determine IgY survival in
vitro in rumen fluid and whether feeding IgY could enhance performance and immune health in lactating Holstein cows.
Chapter 2
EVALUATION OF IGY DISAPPEARANCE DURING IN VITRO INCUBATION IN RUMEN FLUID

Introduction

Administering IgY orally to monogastrics can improve health and performance and benefits may also be observed in ruminants, however, this would be dependent upon passage of IgY out of the rumen to the small intestine. Two separate in vitro experiments were conducted to determine recovery of IgY incubated for various times in rumen fluid. The first objective of Experiment 1 was to determine recovery of IgY from a spray-dried egg product encapsulated with 50% starch (EHE) and IgY from an extract after 0, 0.5 and 6 h of incubation in rumen fluid. We expected higher recovery of IgY from the EHE treatment in rumen fluid compared to IgY from the extract. The second objective was to determine whether the addition of a total mixed ration (TMR) to the in vitro system alters the amount of IgY detected due to additional substrates available for microbial utilization. We hypothesized that IgY detected will decrease over time in rumen fluid but this decrease would be improved with addition of TMR. The purpose of Experiment 2 was to determine the extent of IgY loss from a non-encapsulated spray dried egg product (SDE) prepared from chickens vaccinated against mammalian enteric pathogens used in the in vivo trial (refer to Chapter 3) upon incubation in rumen fluid with TMR over a 24 h time period. Based on the results from Experiment 1, we hypothesized that IgY recovery would continue to decrease over time with minimal recovery at 24 h of incubation.
Materials and Methods

Rumen Fluid Preparation and Buffers

Rumen fluid (~150 mL/cow) was collected in a plastic beaker from the ventral ruminal sac of two lactating ruminally cannulated Holstein cows and pooled. The container was sealed immediately following collection and placed in a jar of warm water for transport to the lab. In the lab, a graduated cylinder was gassed with CO₂ while rumen fluid contents were strained through eight layers of cheesecloth. Rumen fluid was then poured into a beaker maintained at 39.5 °C, gassed with CO₂, and continuously mixed. A reducing solution (0.313 g cysteine HCl, 47.5 mL water, 2 mL 0.1 N NaOH, 0.313 g Na₂S) was added at a ratio of 1 part reducing solution to 5 parts rumen fluid. Trypsinase peptone (BD Biosciences) at 10 mg/mL was then added to the rumen fluid, respectively. Mineral and in vitro buffers were used to simulate buffering that naturally occurs in the rumen in vivo. Mineral buffer solution contained $1.3 \times 10^{-2}$ M Na₂HPO₄, $1.5 \times 10^{-2}$ M KH₂PO₄, $1.7 \times 10^{-3}$ M MgSO₄, $1.7 \times 10^{-4}$ M CaCl₂, $1.1 \times 10^{-4}$ M MnCl₂, $1.1 \times 10^{-5}$ M CoCl₂, $6.2 \times 10^{-5}$ M FeCl₃, pH 7.0, and in vitro buffer contained $5.1 \times 10^{-2}$ M NH₄HCO₃, $4.2 \times 10^{-1}$ M NaHCO₃, pH 7.0. Buffers were maintained at 39.5°C prior to use.

Experiment 1 Treatments

Treatments for experiment 1 included an encapsulated spray-dried egg product (EHE), and an IgY extract (Ext) prepared as a 1:20 dilution of the SDE product in buffer. Solutions containing EHE or extract were prepared by suspending the products in in vitro buffer for a final concentration of 0.33 g/L.
**Experiment 2 Treatments**

The treatment for Experiment 2 included the non-encapsulated spray-dried egg (SDE) product used in the *in vivo* trial (Chapter 3) with TMR incubated in assay buffer (AB; 10 mM PBS supplemented with 0.05% Tween 20, 1% bovine serum albumin, and 0.03% NaN₃ pH 7.4) or rumen fluid. The concentration of the SDE product was estimated based off of the SDE feeding rate from the *in vivo* trial (45 g/d) using a predicted rumen volume of 80 L (Argyle and Baldwin, 1988). Assuming complete consumption of the product immediately following feeding this equates to 0.56 g/L in rumen fluid. This resulted in a final concentration of SDE at 0.108 g/L as the rumen fluid *in vitro* conditions use a ratio of rumen fluid to buffered media of 1:5.2.

**In vitro**

Tubes in experiment 1 were prepared by adding 5 mL of *in vitro* buffer containing EHE or extract and 6 mL of rumen fluid to 15 mL of mineral buffer solution. Assay buffer tubes from experiment 2 were prepared by adding 5 mL of *in vitro* buffer containing SDE to 21 mL of final assay buffer while experimental tubes were prepared by adding 5 mL of *in vitro* buffer containing SDE and 6 mL of rumen fluid to 15 mL of mineral buffer solution. Blanks for both experiments were prepared by adding 6 mL of rumen fluid to 15 mL of mineral buffer and 5 mL of *in vitro* buffer. To serve as a substrate for rumen microbes, 0.3 g of a dried and ground dairy cow TMR containing corn silage, alfalfa silage, hay and concentrate was added to tubes when indicated. Blanks and experimental tubes were gassed with CO₂ while rumen fluid was added to each tube and tubes were immediately capped. In Experiment 1, tubes containing rumen fluid and extract (RF+Ext) were replicated in triplicate and harvested at time points of 0.5 and 6 h and tubes containing rumen fluid, TMR and
extract (RF+TMR+Ext) or encapsulated spray-dried egg (RF+TMR+EHE) were replicated in quadruplicate and harvested at time points of 0, 0.5 and 6 h. Blanks containing rumen fluid only were run individually at each time point. For Experiment 2, tubes containing assay buffer, TMR and spray-dried egg (AB+TMR+SDE) were replicated in triplicate while tubes containing rumen fluid, TMR, and spray-dried egg (RF+TMR+SDE) were replicated in quadruplicate at each time point of 0, 3, 6, 12, 18 and 24 h. The *in vitro* was not replicated for Experiment 1 but was replicated on 4 non-consecutive days for Experiment 2. Experiment 1, n = 1, df = 0. Experiment 2, n = 4, df = 3.

*pH, Incubation and Storage*

Tubes designated for 0 h were immediately flash frozen for 15 minutes in an ethanol/dry ice bath and stored at -80°C. Remaining tubes were incubated at 39.5°C in a shaker incubator and removed after the allotted time. Tubes were flash frozen as described previously and stored at -80°C.

*ELISA*

An enzyme-linked immunoassay (ELISA) was performed for detection of total IgY. 96-well flat bottom microtiter plates (Immunon 2 HB; Thermo Labsystems; Beverly, MA) were coated with 100 µl of borate buffered saline (pH 9.2) containing 10 µl of 25% glutaraldehyde (Sigma Aldrich; St. Louis, MO) and 12.5 µl of primary antibody rabbit anti-IgY F(ab’)2 (Jackson Immunoresearch Laboratories, Inc. West Grove, PA) then incubated for 1 h at 37°C. Following incubation, plates were stored at 4°C overnight. Plates were removed from 4°C the following day and 180 µl of casein-enzymatic hydrolysate (2% casein enzymatic product in 10 mM PBS supplemented
with 0.05% NaN₃; pH 7.4) was added to all wells to prevent non-specific binding. Plates were then incubated for 30 min at 37°C then washed twice with 300 µl of washing solution (10 mM PBS containing 0.05% Tween 20).

Samples were neutralized 1:2 in initial diluent buffer (50 mM Tris supplemented with 0.05% Tween 20, 1% bovine serum albumin, 0.1% ascorbic acid and 0.03% NaN₃; pH 8.25) while final diluent buffer (10 mM PBS supplemented with 0.05% Tween 20, 1% bovine serum albumin and 0.03% NaN₃; pH 7.4) was used for remaining dilutions. Samples for Experiment 2 were diluted as follows: 1:2 → 1:20 → 1:200 → 1:400. Two-hundred microliters of each 1:400 diluted sample was added to duplicate wells. The standard used was chicken IgY (Sigma Aldrich) diluted 1:10 in final assay buffer for an initial concentration of 100 ng/ml then serially diluted 2-fold for a final concentration of 1.56 ng/ml.

Plates were incubated for 1 h at 37°C then washed twice with 300 µl PBS. One-hundred microliters of conjugate diluent (10 mM PBS supplemented with 5% horse serum) containing 125 µl of goat anti-chicken IgY whole antibody diluted 1:40,000 and conjugated to horse-radish peroxidase (HRP) was added to all wells. Plates were incubated for 30 min at 37°C then washed twice 300 µl washing solution. Tetramethylbenzidine (Clinical Science; Mansfield, MA), used as a substrate for HRP, was added to wells and the plate was incubated for 15 min at 37°C. The reaction was stopped with the addition of 100 µl stop solution (1% HCl in DI water) and the plate was read at a wavelength of 450 nm.

Data and Statistical Analysis

For Experiments 1 and 2, recovery was calculated as a percentage of the 0 h samples or as a percentage of the 0 h samples in assay buffer, respectively. The
recovery of IgY was evaluated using the glimmix procedure of SAS. The model for both experiments included the fixed effects of treatment, time and the interaction of time × treatment. Date was also included as a random variable in Experiment 2. Significance was declared at $P \leq 0.05$ and trends declared at $0.05 < P < 0.10$

**Results**

*Experiment 1*

The $P$ values for treatment, time and treatment × time analyzed in Experiment 1 were 0.03, <0.0001, <0.0001, respectively. The average IgY concentration in $\mu$g/mL for the RF+Ext, RF+TMR+EHE, and RF+TMR+Ext treatments at 0.5 and 6 h was 2.23, 0.60, 2.78, 1.70, 2.08 and 1.20, respectively. For the RF+TMR+EHE treatment, the average IgY concentration in $\mu$g/mL. Figure 1 shows the IgY recovery over 0.5 and 6 h incubation in rumen fluid as a percentage of the 0 h samples. Recovery of IgY was not detected in any of the blanks. The RF+Ext and the RF+TMR+Ext treatments were used to determine the extent of proteolytic activity from rumen microbes in the absence or presence of TMR. Recovery of IgY from the RF+Ext treatment at 0.5 h was 97% and significantly decreased to 26% at 6 h suggesting that rumen microbes extensively degraded IgY over time in the absence of TMR. The addition of TMR improved IgY recovery. At 0.5 h, recovery from the RF+TMR+Ext treatment was 90% and significantly decreased to 52% at 6 h of incubation. Encapsulation may have an impact on stability of IgY in the rumen. At 0.5 h, only 66% of IgY was recovered in the RF+TMR+EHE treatment, which differed from recovery of IgY in the RF+TMR+Ext treatment, while only 40% was recovered after 6 h, however, this was not different from the 52% of IgY recovered in the RF+TMR+Ext treatment.
Experiment 2

P values for treatment, time and the interaction between treatment × time analyzed in Experiment 2 all were <0.0001. Figure 2 shows the IgY recovery over 0, 3, 6, 12, 18 and 24 h of incubation in rumen fluid as a percentage of the 0 h samples in assay buffer. Average pH measurements at 0 h for the AB+TMR+SDE, RF+TMR+SDE and the blank without TMR were determined to be 7.74, 7.11, and 7.21, respectively. The average IgY concentration in μg/mL for the 0, 3, 6, 12, 18, and 24 h time points was determined to be 0.98, 1.02, 0.99, 0.68, 0.73, and 0.75, respectively. Recovery of IgY was never detected in any of the blanks. The AB+TMR+SDE treatment was used to determine if anything in the final assay buffer used in the ELISA could lead to degradation of IgY in the absence of rumen fluid. If there was degradation over time of the IgY in assay buffer or if the IgY formed complexes with the TMR, then IgY recovery would be expected to decrease over time with the lowest amount being recovered at 24 h. At 0 h, recovery of IgY was 100%, and numerically increased to 113% at 3 h. Recovery significantly increased from 100% at 0 h to 125% at 6 h of incubation. Recovery of IgY decreased significantly after 6 h to 89% at 12 h but this was not different from recovery at 0 h. Recovery further decreased to 75% at 18 h and was different from 0 h but did not differ from 12 h. Recovery numerically increased from 75% to 88% at 24 h but this was not different from recovery at 0 or 18 h.

The recovery of IgY in the RF+TMR+SDE treatment over 0, 3, 6, 12, 18 and 24 h of incubation in rumen fluid was calculated as a percentage of the 0 h samples in assay buffer. The average IgY concentration in μg/mL for the 0, 3, 6, 12, 18, and 24 h time points was determined to be 0.89, 0.88, 0.28, 0.15, 0.10, and 0.09, respectively. Recovery of IgY at 0 h was 88%, which was not significantly different from recovery
at 0 h in the AB+TMR+SDE treatment. At 3 h incubation, recovery in the RF+TMR+SDE treatment was 90%. This was not different from recovery at 0 h, however, it was significantly different from recovery at 3 h in the AB+TMR+SDE treatment. Recovery in the RF+TMR+SDE treatment decreased significantly to 47% at 6 h, which was significantly different from recovery at 6 h in the AB+TMR+SDE treatment. Recovery further decreased significantly to 25% at 12 h. However, a numeric decrease in recovery was observed at 18 h and 24 h of 15% and 14%, respectively, but these were not different than recovery at 12 h. In Experiment 2, data from the RF+TMR+SDE treatment were used to approximate the amount of IgY that passes to the abomasum. Rate of IgY disappearance (%/h) was estimated as the slope of the regression line of the natural log of IgY recovery vs. time. Since the in vitro was performed in a closed system, a lag in microbial activity likely occurred at the start of the incubation. Also, degradation rates decreased with time, likely due to various factors such as reduced amount of available substrates for microbial utilization, increased production of end products and microbial death. To account for this, the 0, and 24 h time points were dropped, and the disappearance of IgY over time was determined to be 11.5%/h using data from the 3, 6, 12 and 18 h time points. Liquid passage rate from the rumen was approximated to be 13%/h (Casper et al., 1999; Noftsger, 2005; Shin et al., 2012). By using the formula described below, we estimate that approximately 53% of IgY in the SDE product passes to the abomasum:

\[
\text{Passage rate}/(\text{passage rate} + \text{IgY disappearance}) \times 100\% \quad \text{(Bateman, 2004)}.
\]
Discussion

Experiment 1

The treatments for Experiment 1 consisted of RF+Ext, RF+TMR+Ext and RF+TMR+EHE. Since IgY is a protein, we hypothesized that in the RF+Ext treatment rumen microbes would extensively degrade IgY since it was the primary exogenous substrate added. The recovery of IgY decreased significantly from 97% to 26% after 6 h incubation which supported this hypothesis (Figure 1). We hypothesized that the addition of TMR to the RF+Ext treatment would be a closer representation of the in \textit{vivo} rumen environment with other substrates available for microbial utilization resulting in less degradation of IgY. The results supported this hypothesis. The recovery of IgY in this treatment was significantly higher at 52% compared to IgY recovery in the RF+Ext treatment after 6 h incubation (Figure 1). A second hypothesis for Experiment 1 was that the recovery of IgY from the RF+TMR+EHE treatment would be greater than recovery from the RF+TMR+Ext treatment due to encapsulation with 50% starch. This hypothesis was not supported. Recovery of IgY in this treatment was 66% at 0.5 h and 40% at 6 h, significantly and numerically lower, respectively, compared to the RF+TMR+Ext treatment (Figure 1). Encapsulation was developed to stabilize IgY during pelleting at feed mills. Being encapsulated with 50% starch may increase the degradation rate, as starch is highly degradable in the rumen and may not be the best form of encapsulation for use in ruminants. The significant decrease in recovery in the RF+TMR+EHE treatment observed at 0.5 h incubation may be due to increased rates of degradation since the EHE product was protected with 50% starch while the extract did not contain starch. However, the 0.5 h time point was likely still during the lag phase of the in \textit{vitro} and it is questionable that such an extent of
degradation could take place during that time. Regardless, starch encapsulation did not appear to provide protection from rumen degradation, so this product was not considered for further evaluation.

**Experiment 2**

In Experiment 2, we wanted to determine to what extent IgY from the non-encapsulated SDE product used in the *in vivo* trial survives in rumen fluid using incubation times up to and beyond 6 h. Treatments used in Experiment 2 were AB+TMR+SDE and RF+TMR+SDE. We hypothesized that IgY recovery would not change over time in the AB+TMR+SDE treatment and the results supported this hypothesis (Figure 2). Although recovery was significantly higher than 100% at 6 h and lower than 100% at 18 h, we attribute this to experimental error. While the product was continuously mixed upon addition to tubes, it is possible that aggregation of the product during the *in vitro* or aggregation of IgY during preparation of samples for the ELISA resulted in this variation. For the RF+TMR+SDE treatment, we hypothesized that IgY recovery would be similar to the amount recovered from the RF+TMR+Ext treatment from Experiment 1 at the 6 h time point. We also hypothesized that IgY recovery would continue to decrease over time with minimal recovery at 24 h. The results showed that at 6 h of incubation, IgY recovered from the RF+SDE treatment was 47%, comparable to 52% of IgY recovered from the RF+TMR+Ext treatment in Experiment 1. A significant decrease was observed in recovery between 6 h and 12 h (25%) and numerically decreased to 14% at 24 h.

We used the IgY degradation rate from this study and published passage rates to estimate flow of IgY to the abomasum and found that approximately 53% of IgY would be expected to pass to the abomasum *in vivo*. Though it is evident that IgY
degraded over time, the degradation rate may be low enough to deliver an efficacious dose in ruminants. We therefore elected to evaluate the efficacy of this product in vivo as there are limitations to using an in vitro method to evaluate IgY survivability. The in vitro was conducted in a closed system which limits digestibility due to depletion of substrates, accumulation of end products and death of microbes. Another point to consider is that only part of the microbial population is used as microbes may be lost upon rumen fluid collection. Other limitations include potential interaction of IgY with the solid phase.
Chapter 3

**IN VIVO EVALUATION OF THE EFFECT OF A SPRAY-DRIED EGG PRODUCT ON MILK PRODUCTION AND NEUTROPHIL FUNCTION IN LACTATING DAIRY COWS**

Introduction

Oral administration of IgY to monogastrics has been beneficial to overall performance and health. Studies conducted in healthy swine have demonstrated higher weight gains in pigs that received IgY compared to a control group (Song et al., 2012). When swine were orally challenged with *E. coli*, lower fecal scores and higher weight gains were observed in pigs given IgY compared to pigs that received either no treatment or an antibiotic (Owusu-Asiedu et al., 2003). From studies in monogastrics, IgY does have the potential to improve health. The *in vitro* results discussed in Chapter 2 suggested that a sufficient proportion of IgY may pass through the rumen to the small intestine to elicit its biological activity. The proposed mechanism of IgY is that it will act in a similar manner as s-IgA by binding to pathogens at the mucosal level resulting in a reduced inflammatory response. Therefore, it will inhibit the ability of pathogens to outcompete the host’s microbiota as well as colonize the mucosal epithelium. The objectives of this experiment were to determine the effects of a non-encapsulated spray-dried egg product (SDE) on milk production, milk composition, and neutrophil function in healthy, lactating Holstein cows. We hypothesized that SDE would decrease the inflammatory response and preserve energy as would be evidenced by an increase in milk production. In regards to neutrophil function, we hypothesized that attenuation of the inflammatory response by SDE would improve neutrophil
function. We expected an increase in \textit{in vitro} PMN chemotaxis, phagocytosis, and oxidative burst upon an activating stimulus in response to SDE compared to the control.

\textbf{Materials and Methods}

\textit{Animals, Treatments and Sampling}

All procedures were approved by the University of Delaware Agricultural Animal Care and Use Committee. Thirty lactating Holstein cows were housed at the University of Delaware Farm in free stalls, bedded with sand. Cows were individually fed via a Calan gate feeding system (American Calan, Northwood, NH) and were acclimated to the Calan gates for a two week period prior to starting the experimental ration. Animals were fed \textit{ad libitum}, once daily at approximately 0800 hr. The composition of the experimental ration is summarized in Table 3. Feed offered and refused was recorded each day and later used to determine intake. Refusals were removed and weighed each morning and feed offered was adjusted to target for 10\% refusals.

The experiment was conducted as a cross-over design consisting of a 2-wk pre-treatment period and 2, 6-wk treatment periods. Treatments consisted of the TMR top dressed with 45 g/d of commercially available SDE or TMR with no supplementation designated as control (CON). Animals were blocked by parity and randomly assigned to one of two treatment sequences, which were balanced for DIM and milk yield. Cows that were assigned to treatment sequence 1 received CON during period 1 and were then switched to SDE during period 2, whereas, cows assigned to treatment sequence 2 received the treatments in the opposite order. Body weights were recorded
on two consecutive days at the end of the pre-treatment and treatment periods. Body condition score was conducted by two independent people once during the end of the pre-treatment and treatment periods.

Cows were milked twice daily at approximately 0430 and 1600 h with yield recorded at each milking. Milk samples were taken on AM and PM milkings once each week and analyzed by Dairy One Cooperative Inc. (Ithaca, NY) for fat, true protein, lactose, SCC and MUN using a MilkoSCAN System 4000 (Foss North American, Eden Prairie, Minnesota, USA). Samples were calibrated with a Eurochem CL-10 reference analyzer. Additional milk samples (~20 mL) were collected during the end of the pre-treatment period and at the end of weeks 3 and 6 of treatment periods 1 and 2 for detection of IgY by capture ELISA as described in Chapter 2. Samples were preserved with the addition of 60 µl of a 10% (w/w) sodium azide solution and stored at -20°C.

Corn silage, alfalfa silage and TMR were sampled 3 times each week and composited every 2 weeks for nutrient analyses. Concentrate and orchard grass hay were sampled once a week and composited every 2 weeks for nutrient analyses. Nutrient analyses were conducted by Cumberland Valley Analytical Services (Hagerstown, MD) using wet chemistry methods. Samples were analyzed for CP (method 990.03; AOAC, 2006), NDF using α-amylase and sodium sulfite (Van Soest et al., 1991), ADF (method 973.18; AOAC, 2006), starch (Hall, 2009), sugar (Dubois et al., 1956), lignin (Goering and Van Soest, 1970), and ether extract (method 2003.05; AOAC, 2006). Each week, the DM content of corn silage, alfalfa silage, orchard grass hay and concentrate was analyzed in a 60°C forced draft oven for 48 h and used to adjust proportions of the individual feeds in the TMR. The TMR was
subjected to particle size analyses (Table 3) every two weeks using the Penn State Forage Particle Separator (Pennsylvania State University, University Park, Pennsylvania, USA) and physically effective fiber was measured using a Z-box (William H. Miner Agricultural Research Institute, Chazy, New York, USA).

Blood was collected on two consecutive days at the end of the pre-treatment period and at the end of weeks 3 and 6 of each treatment period. On each sampling day, blood was collected in three 10 mL EDTA tubes (Becton Dickinson, Franklin Lakes, NJ) for PMN isolation. Additional blood was collected on the first consecutive day in serum separator tubes (Becton Dickenson, Franklin Lakes, NJ) for potential future analysis of IgY, haptoglobin, serum amyloid A, and LPS-binding protein and Tempus Blood RNA Tubes (Life Technologies, Carlsbad, CA) for potential future whole blood RNA analyses. Tempus and EDTA tubes were kept on ice at all times. Serum separator tubes were kept at room temperature.

Serum samples were centrifuged at 25°C for 30 min at 1,000 × g. Samples were aliquotted into 2 sets of microcentrifuge tubes each containing 1 mL of serum for the potential analysis of acute phase proteins and IgY detection. Serum samples saved for later IgY detection were preserved with the addition of 3 µl of a 10% w/w sodium azide solution for the prevention of microbial contamination and stored at -80°C. Tempus tubes were inverted vigorously for 15 s immediately after blood collection and were stored at -80°C.

Three EDTA tubes were combined into one 50 mL conical tube per cow and centrifuged at 4°C for 45 min at 1,000 × g. Plasma, buffy coats and 2/3 of the red cell packs were removed from each tube. Erythrocytes from the red cell packs were lysed with 18 mL of hypotonic solution (10.6 mM Na₂HPO₄, 2.7 mM NaH₂PO₄) and
inverted vigorously for 40 s. Isotonicity was restored with the addition of 9 mL of a hypertonic solution (10.6 mM Na₂HPO₄, 2.7 mM NaH₂PO₄, 430 mM NaCl) and inverted. Tubes were centrifuged at 4°C for 5 min at 800 × g, supernatant decanted and neutrophil pellets washed twice with 10 mL Hank’s Buffered Saline Solution (HBSS; Mediatech Inc., Manassas, VA) supplemented with 10 mM HEPES buffer at pH 7.4. If erythrocytes were still observed within the pellet, lysing and restoring was repeated in place of the second HBSS wash. After the final centrifugation, supernatant was decanted and PMN were resuspended in 2 mL of assay media (HBSS with 10 mM HEPES and 5% fetal bovine serum). The concentration of PMN was measured using a Beckman Coulter Counter (Beckman Coulter Inc., Fullerton, CA) and concentration adjusted to 2 x 10⁶ cells/mL for assays of neutrophil function. The remaining neutrophil suspension was added into 2 microcentrifuge tubes per cow and centrifuged at 800 × g for 10 min. The supernatant was pipetted off and 500 µl of Trizol was added to each tube and tubes were stored at -80°C for potential future RNA evaluation. Neutrophils were kept on ice throughout the experiment. On average, from 4 random cows sampled on each assay week, isolated cells were 90% neutrophils as measured by differential staining and were 96% viable, as indicated by trypan blue exclusion.

**Neutrophil, Chemotaxis, Phagocytosis and Oxidative Burst**

Chemotaxis was conducted using four 48 well chambers (Neuro Probe Inc., Gaithersburg, MD). The bottom wells contained 28 µl of assay media only or assay media supplemented with recombinant human interleukin-8 (IL-8; 100 ng/mL; Sigma-Aldrich Co., St. Louis, MO). Polycarbonate membranes (Neuro Probe Inc., Gaithersburg, MD) with 3 µm pores were placed over the bottom wells. Fifty
microliters of PMN suspension (2 × 10^6 mL) was added to the top wells and the chambers were incubated for 1 h at 37°C in atmospheric conditions. All cow and chemoattractant combinations were measured in triplicate wells. After incubation, chambers were disassembled, membranes were removed and dipped in phosphate buffered saline (PBS) three times. Non-migrated cells were scraped off the tops of membranes after each dip. The bottoms of the membranes were dipped in methanol, allowed to dry and were stained (HEMA 3 Stain Set; Fisher Scientific, Kalamazoo, MI). Neutrophil migration towards media alone or towards IL-8 was determined by averaging the number of cells adhered to 5 random fields/well under a 40 × objective.

Oxidative burst was carried out in a 96 well luminometer plate. One-hundred microliters of neutrophils (2 × 10^6 cells/mL) was added to triplicate wells with one well designated as a non-activated control containing 80 μl of assay media. To activate cells, 80 μl of assay media containing activated zymosan (Sigma-Aldrich), prepared according to Rinaldi et al. (2007), was added to the remaining two wells. Activated zymosan stimulates the mannose-fucosyl glycosylated protein receptors. Twenty microliters of luminol was pipetted into all wells for a final concentration of 0.5 mM. The plate was incubated in a Gen51.05 Synergy 2 microplate reader (BioTek Instruments Inc., Winooski, VT) for 2 h at 37°C with luminescence readings recorded every 5 min. Reactive oxygen species production was calculated in arbitrary units as the sum of luminescence measurements over the 2 h incubation period for unstimulated and stimulated PMN.

To assess phagocytosis, carboxylated fluorescent latex beads (Fluoresbrite Yellow Green Carboxylate Microspheres, 1.00 μm, no. 17154; Polysciences Inc., Warrington, PA) were used. Beads were incubated in pooled cow serum in the dark at
room temperature for 45 min. Two-hundred fifty microliters of a $2 \times 10^6$ cells/mL PMN suspension was added to triplicate wells of a 96 well plate followed by addition of 27.5 µl of bead suspension resulting in a 1:10 ratio of neutrophils to beads. The plate was incubated in the dark for 2 h at 37°C in atmospheric conditions. After incubation, the plate was centrifuged at 1,500 × g for 5 min at 4°C and supernatant was decanted. The plate was washed twice with 200 µl of PBS. The supernatant was decanted and 200 µl of a 1% paraformaldehyde solution in PBS was added to all wells. The plate was incubated at room temperature for 15 min to allow for fixation of neutrophils. After incubation, the plate was centrifuged for 5 min at 1500 × g, supernatant decanted and PMN were resuspended in tubes with 600 µl PBS. Four random cows served as controls in which 250 µl of PMN suspension was placed into 4 separate wells. Two out of the four wells contained only neutrophils while the remaining two wells contained neutrophils with bead suspension added following the fixation step. Two additional wells contained beads alone. Figure 3 shows an image of a neutrophil that has engulfed fluorescent beads. The tubes were covered in parafilm, wrapped in aluminum foil and stored at 4°C until flow cytometer analysis (FACSCalibur, BD Biosciences). Flow cytometry was conducted using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The population was gated for neutrophils based on forward and side scatter, and 4,000 events within the neutrophil gate were counted for each sample (Figure 4; Nemec et al., 2012). A fluorescence histogram was used to differentiate neutrophils that had engulfed ≥1 bead from neutrophils that did not engulf beads (Figures 5-7). The control tubes were used to set the neutrophil gate, set the recording regions of the fluorescence histogram, and to correct samples for nonspecific fluorescence. Flow cytometry did not differentiate
between cells that were viable and nonviable at the time of paraformaldehyde addition. Results were expressed as the percentage of total neutrophils containing either 1 bead or > 1 bead.

**Data and Statistical Analysis**

The experiment initially started with 6 primiparous and 24 multiparous cows. As the study progressed, 1 primiparous and 2 multiparous cows were removed due to illness or failure to train to the Calan gates. Another 2 multiparous cows were removed from the data set prior to analysis due to low milk yields. Data from the 25 remaining cows were evaluated using the glimmix procedure of SAS. The model included the fixed effects of treatment (Control or SDE), sequence (1 or 2), period (1 or 2), parity (1 or >1), and the interaction of treatment × parity. Data collected during the pre-treatment period were included as covariates. For repeated measures data, the model also included fixed effects of week (1 to 6 for production variables and 3 or 6 for neutrophil measures) and the interaction of treatment × week, and covariance was modeled using the first-order autoregressive structure. Significance was declared at $P \leq 0.05$ and trends declared at $0.05 < P \leq 0.10$.

**Results**

The nutrient composition of the ration is summarized in Table 3. The ration was formulated to contain 16.5% CP, 31.2% NDF and 25.0% starch, and analyzed concentrations were 16.1% CP, 31.6% NDF, and 22.8% starch. The ELISA did not detect IgY in serum or milk samples. Table 4 shows $P$ values for treatment effects on production measures. There was no main effect of treatment or treatment × week on any of the production measures, however, there was a trend for an interaction between
treatment × parity on milk yield \( (P = 0.10; \text{Figure 3}) \). The SDE treatment numerically decreased milk yield in primiparous cows by 1.65 k/d while it numerically increased milk yield in multiparous cows by 0.30 k/d. There was also a trend for an interaction between treatment × parity on milk/DMI \( (P = 0.06; \text{Figure 4}) \). A numerical decrease in milk/DMI in response to SDE was observed in primiparous cows while milk/DMI numerically increased in multiparous cows suggesting SDE increased efficiency in multiparous cows but decreased efficiency in primiparous cows.

\( P \) values for treatment effects on milk components are summarized in Table 5. There was an effect of treatment on fat yield \( (1.72 \text{ kg/d control vs } 1.67 \text{ kg/d SDE}; P = 0.05) \), brought about by primiparous cows as evidenced by an interaction between treatment × parity \( (P = 0.03) \) (Figure 5). The SDE treatment numerically decreased fat yield in primiparous cows while fat yield in multiparous cows remained unaffected. There was also an effect of treatment × parity on protein percent \( (P = 0.01; \text{Figure 7}) \). In response to the SDE treatment, protein percent numerically increased in primiparous cows, but decreased in multiparous cows. A trend for an interaction between treatment × week on lactose percent was observed \( (P = 0.08; \text{Figure 8}) \). Lactose percent in response to SDE was significantly lower than the control at wk 3.

As for measures of neutrophil function, there was a trend for a treatment effect on chemotaxis towards media only (Table 6). In response to SDE, PMN migration towards media alone numerically increased compared to migration towards the control \( (P = 0.09) \). There was a numeric increase in migration towards IL-8 in response to SDE compared to migration towards the control \( (P = 0.51) \). A main effect of treatment was not observed on oxidative burst production in unstimulated PMN. There was a trend for an interaction between treatment × week on oxidative burst production in
stimulated PMN \((P = 0.10)\). Oxidative burst in stimulated PMN in response to SDE during wk 3 was numerically lower than the control by 2882 AU, however, it was numerically higher than the control by 1606 AU at wk 6 (Figure 9). Neutrophil phagocytosis was unaffected by treatment.

**Discussion**

The target site for IgY in the SDE product used in the *in vivo* trial was at the level of the small intestine. Immunoglobulin Y is proposed to act by binding to pathogens within the lumen thereby preventing pathogens from adhering to and colonizing the mucosa. We hypothesized that the SDE product would increase milk yield by attenuating inflammatory responses at the gut level, thus, conserving energy. However, the results do not support this hypothesis as a main effect of treatment on milk yield was not observed (Table 4). There were also no main effects of treatment on production measures of DMI, and Milk/DMI. On the contrary, interactions observed in treatment \(\times\) parity on milk yield and milk/DMI suggest that primiparous cows had poorer performance in response to SDE compared to primiparous cows on control. Our results are in contrast to those of Ibarbia et al. (2014) who reported an interaction between week \(\times\) treatment on milk yield in lactating dairy cows fed 3 g of a commercially developed avian derived polyclonal antibody preparation against LPS (PAP-LPS) for the first 14 d post-calving. That group observed a significant increase in milk yield during wks 3, 4, 6, and 10 in cows fed PAP-LPS compared to the control (Ibarbia et al., 2014). Their study included a larger number of cows (n = 400), suggesting that our study may have been under-replicated. In our study, cows were on treatment for 6 wks beginning on average at 85 DIM, yet no significant differences were observed in milk yield, while in the study conducted by Ibarbia et al. (2014)
cows were on treatment from the day of calving until 14 DIM, however, differences in milk yield were observed well beyond the end of the supplementation period. These results suggest that SDE treatment may be beneficial when given shortly after calving when cows commonly experience suppressed immune function. Another point to consider is that our study did not induce a diet shift as cows were acclimated to a transition diet prior to administering SDE. Administering the PAP-LPS preparation may have reduced the inflammatory response triggered by the change in diet by altering species abundance or toxin production.

The current dose administered to horses is 30 g which was approximated by multiplying the human dose of 4.5 g by 6.86, the ratio of the average body weight of horses to that of humans. To account for a larger body weight in cows, the dose was raised to 45 g, which was top-dressed daily on the TMR. This product contained approximately 8 mg/g of total IgY, which equated to approximately 360 mg of total IgY against various human enteric pathogens assuming full consumption of the product. We estimated that approximately 53% of IgY would be expected to pass to the abomasum in vivo which roughly equates to 190 mg of IgY reaching the abomasum. Rams (39 kg) were orally inoculated with $10^{10}$ CFU of *E. coli* and then orally administered 100 g of a spray-dried egg preparation from hens immunized with *E. coli* O157:H7. The dose equated to approximately 1000 mg of IgY with 100 mg of IgY specific for *E. coli* (Cook et al., 2005). The 100 g dose significantly reduced fecal shedding of *E. coli* compared to a dose of 25 g and control and numerically reduced shedding compared to a 50 g dose. This group administered a dose 2.2 times greater to animals with a 19 fold difference in weight compared to the dose (45 g) and average
weight (726 kg) for the animals used in our study. This suggests that the dose used in our study was not efficacious to elicit a change.

While dose is important, treatment preparation may need to be addressed, specifically, whether or not the product should be prepared against a variety or only a few specific pathogens. The SDE product used in our study was produced from whole eggs and contained IgY against various human enteric pathogens but no differences were observed in performance or neutrophil function. On the contrary, the product used in the Ibarbia et al. (2014) study was specific against LPS and differences in milk yield were observed well beyond the end of the supplementation period. Similarly, Cook et al. (2005) used a product specific against *E. coli* and demonstrated reduced fecal shedding of this pathogen upon challenge. In contrast to Cook et al. (2005), cows in our study were not challenged and it may be worthwhile to really demonstrate the potential benefits of IgY in challenged animals. An example of a challenge could be acidosis considering that it is a common health concern among dairy cows. With the results from these studies, it is likely that an antibody prepared against opportunistic commensals known to cause disease may be more beneficial in improving overall performance and health.

Studies conducted in other species reveal that production and health benefits of IgY preparations are dose dependent and that too much or too little can have positive or negative health effects. Nguyen et al. (2005) found that dogs who received 2 g of IgY powder against canine parvovirus were better protected upon an oral challenge than dogs who received only 0.5 g. Shin et al. (2002) administered a daily oral dose of either 1 mg or 10 mg of IgY against *H. pylori* to 6 wk old Mongolian gerbils and also observed a reduction in mucosal injury in gerbils who received the higher dose due to
decreased numbers of adhered *H. pylori*. Hong et al. (2004) conducted a growth study in pigs assigned to diets consisting of either 3% or 6% of spray-dried egg protein containing specific egg yolk antibodies (SDEP) against *E. coli* or a diet containing no SDEP. This group observed that as the percentage of SDEP increased in the diet, feed intakes and weight gains numerically decreased (Hong et al., 2004). However, in a separate experiment conducted by Hong et al. (2004), this group observed that pigs who received diets supplemented with 0.2% SDEP against *E. coli* had numerically higher average daily gains than pigs who received the same diet supplemented with 0.1% SDEP. Chernysheva et al. (2003) assigned 3 wk old pigs to diets containing either 3.2 g/kg (0.32% of the diet), 32 g/kg (3.2% of the diet), or no egg yolk antibody from hens vaccinated against fimbrial antigens K88, K99, and K87P of *E. coli*. Pigs were then orally challenged with $5 \times 10^{11}$ CFU of *E. coli* strain 0149 K88ac+. Pigs assigned to diets containing either dose of the egg yolk antibody were not protected against the challenge and showed signs of disease (Chernysheva et al., 2003). In summary, IgY can improve performance and health if the correct dose is administered.

Immune function is altered during systemic inflammation resulting in increased susceptibility to disease. In regards to assessing PMN function *in vitro*, we hypothesized that SDE treatment would ameliorate systemic inflammation as would be evidenced by enhanced neutrophil function *in vitro*. Circulating PMN function during systemic inflammation *in vitro* is reduced upon an activating stimulus. We expected to see a more robust response of PMN chemotaxis, oxidative burst, and phagocytosis in response to SDE treatment compared to control upon the addition of an activating stimulus. The results do not support this hypothesis as there were no main effects of treatment on chemotaxis, oxidative burst, or phagocytosis. This
supports the findings of Ibarbia et al. (2014) in that no significant differences were observed in disease incidence or serum acute phase proteins in cows given PAP-LPS compared to cows on control. However, this contradicts findings from other species. Zhen et al. (2011) found that mice intraperitoneally injected with 200 mg/kg of specific IgY against *E. coli* 0111 and challenged with 10 mg/kg LPS through intraperitoneal injection had decreased serum levels of TNF-α 6 h and increased IL-10 2 h post LPS challenge compared to the control (Zhen et al., 2011). While it is not practical to inject large numbers of cows, their study does demonstrate that specific IgY targeted against *E. coli* can affect circulating levels of pro and anti-inflammatory cytokines in response to LPS challenge.

Evidence from studies of inflammatory conditions has demonstrated that circulating PMN are primed, however, PMN effector functions in vitro is altered in response to an activating stimulus. Even though chemotaxis towards media only numerically increased in response to SDE, there was no effect of treatment on chemotaxis towards IL-8. This contrasts findings from Aglas et al. (1998) who demonstrated that when PMN from RA patients was stimulated with fMLP, a significant reduction in migration was observed, while migration significantly increased in PMN from healthy individuals. We expected to see enhanced migration towards IL-8 in response to SDE. Oxidative burst from our study was not affected by SDE. Curran et al. (1991) reported that superoxide production in stimulated PMN from Crohn’s disease patients was significantly lower compared to healthy controls. Trottier et al. (2012) also observed a significant decrease in superoxide production in stimulated PMN from obese individuals compared to controls. Our lack of treatment
effect observed on oxidative burst of PMN suggests that the dose may not have been efficacious to elicit a change.

Conclusions and Future Direction

The SDE treatment did not have an effect on performance or PMN functions measured \textit{in vitro}. It is suggested from Chapter 2 that IgY does survive in rumen conditions with an estimated 53\% passing to the abomasum. Shifts in the microbial composition of the gut are implicated in generating innate or adaptive immune problems within systemic circulation. The SDE product should be made to target one or more species of endemic commensal organisms known to increase within the bovine commensal population when diet shifts occur. To identify which commensals are impacted during a diet shift, an acidosis challenge can be performed. The product needs to be able to survive passage from the abomasum while retaining sufficient antigenic reactivity. A passage rate experiment should be conducted to determine the rate of passage and an ELISA can be used to quantify the antigenic reactivity of the product. If a beneficial response is found in the future, other work can include dose-response studies to determine the efficacious dose or what the required minimum dose is to elicit a beneficial change in the animal.
Table 3. Ingredient and chemical composition of the total mixed ration including particle size and physically effective fiber measurements.

<table>
<thead>
<tr>
<th><strong>Ingredient</strong></th>
<th>% of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>54.39</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>8.19</td>
</tr>
<tr>
<td>Orchard grass hay</td>
<td>2.87</td>
</tr>
<tr>
<td>Corn grain, ground</td>
<td>9.64</td>
</tr>
<tr>
<td>Turbo meal</td>
<td>6.41</td>
</tr>
<tr>
<td>Canola meal</td>
<td>5.01</td>
</tr>
<tr>
<td>Citrus pulp, dry</td>
<td>5.8</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>1.47</td>
</tr>
<tr>
<td>Porcine blood meal</td>
<td>0.93</td>
</tr>
<tr>
<td>Rumen Bypass Fat</td>
<td>1.09</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>0.79</td>
</tr>
<tr>
<td>Palm Fat C:16</td>
<td>0.66</td>
</tr>
<tr>
<td>Trace mineral and vitamin mix</td>
<td>0.41</td>
</tr>
<tr>
<td>Biophosphate</td>
<td>0.24</td>
</tr>
<tr>
<td>Slow release urea</td>
<td>0.18</td>
</tr>
<tr>
<td>Urea</td>
<td>0.29</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>0.29</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>0.35</td>
</tr>
<tr>
<td>Calcium Sulfate Dihyd</td>
<td>0.11</td>
</tr>
<tr>
<td>Potassium Carbonate</td>
<td>0.14</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>0.11</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.03</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.01</td>
</tr>
<tr>
<td>Smartamine M</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**Chemical composition % of DM**

<table>
<thead>
<tr>
<th>Component</th>
<th>% of DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>47.6</td>
</tr>
<tr>
<td>NE_{L} (mcal/kg)</td>
<td>1.8</td>
</tr>
<tr>
<td>TDN</td>
<td>72.6</td>
</tr>
<tr>
<td>CP</td>
<td>16.1</td>
</tr>
<tr>
<td>NDF</td>
<td>31.6</td>
</tr>
<tr>
<td>ADF</td>
<td>21.3</td>
</tr>
<tr>
<td>Starch</td>
<td>22.8</td>
</tr>
<tr>
<td>Ash</td>
<td>7.8</td>
</tr>
<tr>
<td>Ca</td>
<td>0.8</td>
</tr>
<tr>
<td>P</td>
<td>0.4</td>
</tr>
<tr>
<td>Mg</td>
<td>0.5</td>
</tr>
<tr>
<td>K</td>
<td>1.3</td>
</tr>
<tr>
<td>NFC</td>
<td>41</td>
</tr>
<tr>
<td>NSC</td>
<td>28.5</td>
</tr>
<tr>
<td><strong>Penn state particle separator</strong>&lt;sup&gt;5&lt;/sup&gt;</td>
<td><strong>% retained</strong></td>
</tr>
<tr>
<td>Upper sieve</td>
<td>3.66</td>
</tr>
<tr>
<td>Middle sieve</td>
<td>38.65</td>
</tr>
<tr>
<td>Lower sieve</td>
<td>38.33</td>
</tr>
<tr>
<td>Bottom pan</td>
<td>19.38</td>
</tr>
<tr>
<td><strong>Z-box</strong>&lt;sup&gt;6&lt;/sup&gt;</td>
<td><strong>%</strong></td>
</tr>
<tr>
<td>Physically effective fiber</td>
<td>0.6120</td>
</tr>
</tbody>
</table>

<sup>1</sup>Rumen Bypass Fat: MegaLac (Church & Dwight Co., Inc, Princeton, NJ)
<sup>2</sup>Trace mineral and vitamin mix: 32.0% Mg, 7.6% S, 4.5% K, 2.0% Ca, 12,000 mg/kg Zn, 6,000 mg/kg Mn, 2,250 mg/kg Cu, 1,900 mg/kg Fe, 218 mg/kg I, 164 mg/kg Co, 87 mg/kg Se, 1,411 IU/g Vitamin A, 353 IU/g Vitamin D, 7 IU/g Vitamin E
<sup>3</sup>Slow release urea: Optigen (Alltech Inc., Nicholasville, KY)
<sup>4</sup>DCAD Plus (Church & Dwight Co., Inc, Princeton, NJ)
<sup>5</sup>Penn State Forage Particle Separator (Pennsylvania State University, University Park, Pennsylvania, USA)
<sup>6</sup>Z-box (William H. Miner Agricultural Research Institute, Chazy, New York, USA).
Table 4. Effect of treatment on production measures.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>SDE</th>
<th>SEM</th>
<th>Covariate</th>
<th>Parity</th>
<th>Treatment</th>
<th>Treatment×Week</th>
<th>Treatment×Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk, kg/d</td>
<td>45.16</td>
<td>44.48</td>
<td>1.67</td>
<td>0.03</td>
<td>0.07</td>
<td>0.25</td>
<td>0.51</td>
<td>0.10</td>
</tr>
<tr>
<td>DMI, kg/d</td>
<td>27.19</td>
<td>27.14</td>
<td>0.64</td>
<td>&lt;0.0001</td>
<td>0.10</td>
<td>0.92</td>
<td>0.42</td>
<td>0.49</td>
</tr>
<tr>
<td>Milk/DMI</td>
<td>1.70</td>
<td>1.69</td>
<td>0.05</td>
<td>&lt;0.0001</td>
<td>0.95</td>
<td>0.42</td>
<td>0.44</td>
<td>0.06</td>
</tr>
<tr>
<td>BW, kg</td>
<td>731</td>
<td>733</td>
<td>7.52</td>
<td>&lt;0.0001</td>
<td>0.39</td>
<td>0.76</td>
<td>-</td>
<td>0.43</td>
</tr>
<tr>
<td>BCS</td>
<td>2.95</td>
<td>2.98</td>
<td>0.08</td>
<td>0.0004</td>
<td>0.08</td>
<td>0.60</td>
<td>-</td>
<td>0.81</td>
</tr>
</tbody>
</table>

1 Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control.

Table 5. Effect of treatment on milk composition.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>SDE</th>
<th>SEM</th>
<th>Covariate</th>
<th>Parity</th>
<th>Treatment</th>
<th>Treatment×Week</th>
<th>Treatment×Parity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat, %</td>
<td>3.78</td>
<td>3.70</td>
<td>0.09</td>
<td>0.04</td>
<td>0.10</td>
<td>0.19</td>
<td>0.25</td>
<td>0.31</td>
</tr>
<tr>
<td>Fat, kg/d</td>
<td>1.72</td>
<td>1.67</td>
<td>0.06</td>
<td>0.0012</td>
<td>0.12</td>
<td>0.05</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>Protein, %</td>
<td>2.86</td>
<td>2.86</td>
<td>0.04</td>
<td>&lt;0.0001</td>
<td>0.84</td>
<td>0.89</td>
<td>0.57</td>
<td>0.01</td>
</tr>
<tr>
<td>Protein, kg/d</td>
<td>1.31</td>
<td>1.29</td>
<td>0.04</td>
<td>0.001</td>
<td>0.24</td>
<td>0.21</td>
<td>0.69</td>
<td>0.25</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>4.71</td>
<td>4.69</td>
<td>0.03</td>
<td>&lt;0.0001</td>
<td>0.55</td>
<td>0.26</td>
<td>0.08</td>
<td>0.88</td>
</tr>
<tr>
<td>Log SCC</td>
<td>2.02</td>
<td>1.96</td>
<td>0.09</td>
<td>&lt;0.0001</td>
<td>0.15</td>
<td>0.43</td>
<td>0.52</td>
<td>0.90</td>
</tr>
<tr>
<td>MUN, mg/dL</td>
<td>13.28</td>
<td>13.40</td>
<td>0.33</td>
<td>0.001</td>
<td>0.15</td>
<td>0.65</td>
<td>0.63</td>
<td>0.63</td>
</tr>
</tbody>
</table>

1 Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control.
Table 6. Effect of treatment on neutrophil function assays of chemotaxis, oxidative burst and phagocytosis.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Treatment¹</th>
<th>P - values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SDE</td>
</tr>
<tr>
<td>Chemotaxis², #/field, media only</td>
<td>21.67</td>
<td>26.19</td>
</tr>
<tr>
<td>Chemotaxis³, #/field, IL-8</td>
<td>57.63</td>
<td>60.42</td>
</tr>
<tr>
<td>Oxidative⁴ burst, AU, unstimulated</td>
<td>269</td>
<td>259</td>
</tr>
<tr>
<td>Oxidative⁵ burst, AU, stimulated</td>
<td>22180</td>
<td>21532</td>
</tr>
<tr>
<td>Phagocytosis⁶, %, 1 bead</td>
<td>18.41</td>
<td>18.03</td>
</tr>
<tr>
<td>Phagocytosis⁷, %, &gt; 1 bead</td>
<td>45.30</td>
<td>45.46</td>
</tr>
</tbody>
</table>

¹Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control.
², ³Neutrophil migration through a 3-µm pore membrane towards media only or media supplemented with human IL-8 (100 ng/mL) was measured following 1-h incubations as average number of cells per 5 microscope field und 40x objective.
⁴-⁵Neutrophils were unstimulated or stimulated with zymosan and incubated with 0.5 mM luminol. Oxidative burst was measured as the sum of luminescence (arbitrary units) every 5 min for 2 h.
⁶-⁷Phagocytosis is presented as the percent of total neutrophils that engulfed 1 or > 1 fluorescent carboxylated latex beads following 2 h incubations.
Figure 1  Interaction between sample × time on IgY recovery from rumen fluid in Experiment 1. Treatments included rumen fluid+extract (RF+Ext), rumen fluid+TMR+extract (RF+TMR+Ext) and rumen fluid+TMR+encapsulated spray-dried egg (RF+TMR+EHE). IgY recovery (%) was calculated as a percentage of the 0 h samples. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ (p < 0.0001).
Figure 2  Interaction between sample × time on IgY recovery ($P < 0.0001$) from rumen fluid in Experiment 2. Treatments included assay buffer+TMR+spray-dried egg (AB+TMR+SDE) and rumen fluid+TMR+spray-dried egg (RF+TMR+SDE). IgY recovery (%) was calculated as a percentage of the 0 h samples in assay buffer. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ ($p < 0.0001$).  

![Figure 2](image URL)

Figure 3  Neutrophil that has engulfed 1.00 μm fluoresbrite yellow green carboxylate beads. A slide was prepared and neutrophils were stained (HEMA 3 Stain Set; Fisher Scientific, Kalamazoo, MI) and viewed under a 40 × objective for the presence of bead engulfment.
Figure 4  Scatter plot of the neutrophil control showing the gate around the neutrophil population. Flow cytometry was conducted using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The population was gated for neutrophils based on forward and side scatter, and 4,000 events within the neutrophil gate were counted for each sample.
Figure 5  Fluorescence histogram plot of the neutrophil control. Flow cytometry was conducted using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The population was gated for neutrophils based on forward and side scatter, and 4,000 events within the neutrophil gate were counted for each sample. A fluorescence histogram was used to differentiate neutrophils that had engulfed ≥1 bead from neutrophils that did not engulf beads.
Figure 6  Fluorescence histogram plot of the bead control. Flow cytometry was conducted using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The population was gated for neutrophils based on forward and side scatter, and 4,000 events within the neutrophil gate were counted for each sample. A fluorescence histogram was used to differentiate neutrophils that had engulfed ≥1 bead from neutrophils that did not engulf beads with the placement of markers (M).
Figure 7  Fluorescence histogram plot of the neutrophil and bead suspension control. Flow cytometry was conducted using an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The population was gated for neutrophils based on forward and side scatter, and 4,000 events within the neutrophil gate were counted for each sample. A fluorescence histogram was used to differentiate neutrophils that had engulfed ≥1 bead from neutrophils that did not engulf beads with the placement of markers (M). Results were expressed as the percentage of total neutrophils containing either 1 bead or > 1 bead.
**Figure 8**  Trend for an interaction between treatment × parity on milk yield ($P = 0.10$). Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ.

**Figure 9**  Trend for an interaction between treatment × parity on milk yield divided by dry matter intake ($P = 0.06$). Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ.
Figure 10  Interaction between treatment × parity on milk fat yield ($P = 0.03$). Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ.

Figure 11  Interaction between treatment × parity on milk protein percentage ($P = 0.01$). Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ.
Figure 12  Trend for an interaction between treatment × week on milk lactose percent ($P = 0.08$). *declares significance at ($P < 0.05$). Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ.

Figure 13  Trend for an interaction between treatment × week on neutrophil production of reactive oxygen species ($P = 0.10$). Treatments consisted of a TMR top-dressed with 45 g/d of whole spray-dried egg (SDE) or TMR with no supplementation designated as control. Error bars indicate SED for treatment × time. a,b,c,d Bars with unlike letters differ.
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