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Assessment of a Nutritional Rehabilitation Model in Two Modern Broilers and Their Jungle Fowl Ancestor: A Model for Better Understanding Childhood Undernutrition

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Assessment of a Nutritional Rehabilitation Model in Two Modern Broilers and Their Jungle Fowl Ancestor: A Model for Better Understanding Childhood Undernutrition

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by

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Abstract

The World Health Organization, estimated that 22.9% of children under the age of 5 are stunted. The etiology of stunting is multifactorial and is associated with poor linear growth, villous atrophy, dysbiosis, and increased intestinal permeability. Inclusion of rye in poultry diets induces nutrient deficiencies and increases intestinal permeability, dysbiosis and decreases growth rates. The objective of this dissertation was to determine if chickens consuming a rye based diet exhibited a similar pathophysiology of stunted children to develop a relevant animal model. Therefore, early or late phase malnutrition was induced determine the effects of malnutrition on performance, bone mineralization, intestinal morphology and paracellular intestinal leakage across three diverse genetic backgrounds. 2015 Cobb chicken, 1995 Cobb chicken, and the Jungle Fowl were allocated into four different dietary treatments. Dietary treatments were (1) a control corn-based diet throughout the trial (corn–corn); (2) an early phase malnutrition diet where chicks received a rye-based diet for 10 days, and then switched to the control diet (rye–corn); (3) a malnutrition rye-diet that was fed throughout the trial (rye–rye); and (4) a late phase malnutrition diet where chicks received the control diet for 10 days, and then switched to the rye diet (corn–rye). Modern broilers in the rye-corn treatment group exhibited catch up growth and was able to fully recover all of the growth and bone parameters measured after the consumption of a rye based diet. However, the rye-corn group was unable to recover was the serum FITC-D indicating the gut was still leaky. 1995 broilers in the rye-corn group had significantly lower BW, BWG, and tibia strength and higher serum FITC-D than the corn-corn group indicating that these birds were not able to fully recover within the observed timeframe. Jungle fowl appeared to have a higher tolerance to the rye based diet, as there were minimal differences between dietary treatments for the parameters measured. This suggests that a rye-

based diet is a viable approach to induce malnutrition in chickens and slower compensatory growth rate observed in the 1995 broilers was similar to that of stunted children in developing countries.

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Dedication

I would like to dedicate my dissertation to my family, friends and my partner Jacob. My family and friends were so supportive of my decision to move to Arkansas and have been really supportive throughout my time here. My parents are exceptionally amazing people and I really owe everything I have to them. To my family in Arkansas, have made me feel welcomed and loved and I am forever indebted to “all y’all”. Lastly, Jacob you were part of the reason I chose to attend the University of Arkansas and I will be thanking you for the rest of our lives together.

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Baxter, M. F., R. Merino-Guzman, J. D. Latorre, B. D. Mahaffey, Y. Yang, K. D. Teague, L. E. Graham, A. D. Wolfenden, X. Hernandez-Velasco, L. R. Bielke, and others. 2017. Optimizing Fluorescein Isothiocyanate Dextran Measurement As a Biomarker in a 24-h Feed Restriction Model to Induce Gut Permeability in Broiler Chickens. Frontiers in Veterinary Science 4. (Chapter 2).

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Baxter, M. F., J. D. Latorre, D. A. Koltes, S. Dridi, E. S. Greene, S. W. Bickler, J. H. Kim, R. Merino-Guzman, X. Hernandez-Velasco, N. B. Anthony, and others. 2018. Assessment of a Nutritional Rehabilitation Model in two modern broilers and their Jungle fowl ancestor: Evaluation of gut barrier function. To be submitted to Frontiers in Physiology. (Chapter 4).

Introduction

In 2016, World health organization (WHO), estimated that 155 million or 22.9% of children under the age of 5 are stunted and 52 million or 7.7% of children under the age of 5 are wasted. Stunting is described as low height for a particular age and is often reflected in poor linear growth (Victora et al., 2010; Prendergast and Humphrey, 2014), while wasting is low weight for a particular height (Victora et al., 2010). The increased risk of mortality is the short term effects of stunting while long term consequences include reduced cognitive ability which reduce learning and increased risk of metabolic diseases (Mbuya and Humphrey, 2016; Black et al., 2017; Wang et al., 2017). From conception until 2 years old is when children are the most vulnerable to stunting (De Onis et al., 2012) and this is the time period when interventions are the most effective (Victora et al., 2010). The etiology of stunting is multifactorial and includes undernutrition, infection, environmental enteropathy (EE), poor health care and improper sanitation (Seetha et al., 2018). Children are typically unable to exhibit full catch up growth after a period of undernutrition (Golden, 1994). The ability the fully recover growth lost during malnutrition may be dependent on the severity of the insults and the environment required to support growth (Golden, 1994).

In Malawi, one of the least developed countries in the world, had a 42% rate of stunting (Kanyuka et al., 2016). One of the primary causes of this stunting is micro and macro nutrient deficiencies where 70% of the diets consists of nisma which is a hard porridge consisting of maize (Mlotha et al., 2016). This starchy grain lacks micro and macronutrients required to support the development of a child and is susceptible to aflatoxin growth (Seetha et al., 2018). The addition of other grains and legumes would complement the nutrient profiles of maize, however there is limited education on diet diversification and culturally nisma is a traditional

food item (Seetha et al., 2018). Furthermore, malabsorption and diarrhea is often caused by unsafe drinking water and inadequate hygiene and sanitation. Seetha et al., (2018) implemented comprehensive training interventions targeting diet diversification and increased sanitation practiced. There was a significant decrease in the severity of wasting and undernutrition, however training did not significantly reduce the incidence of stunting over a 21 day period (Seetha et al., 2018). Perhaps significant differences may have been observed if these training and monitoring sessions continued over a longer period of time (Seetha et al., 2018). The incidence of diarrhea improved greatly when proper sanitation practices were implemented and was correlated with overall improvements in growth (Seetha et al., 2018). Therefore, improving the nutrient profiles of the diet and reducing EE decreased the occurrence of diarrhea and may reduce the severity of stunting if implemented over a longer period of time (Seetha et al., 2018). This study illustrates the interaction between nutrient and microbial profiles with growth.

The poultry industry was the first to document that chicks in unsanitary conditions were able to attain improved growth rates when antibiotics were added to the feed (Solomons et al., 1993). Stimulation of the immune system induces metabolic changes in chickens which divert nutrients from growth and favor a metabolic process that supports immune response and disease resistance (Solomons et al., 1993). Antibiotics have been hypothesized to increase growth by decreasing the number and severity of bacterial infections which trigger immune stimulation (Solomons et al., 1993) and perhaps attenuating the immune response (Niewold, 2007). Therefore, antibiotics may be masking unsanitary conditions in which a chicken lives in and the removal of antibiotics may trigger similar pathophysiology observed in patients with EED.

From previous studies it is also evident that chickens consuming a rye-based diet have some of the same pathophysiological conditions as those stunted children including poor growth rates, a reduction in bone quality, dybiosis and increased intestinal permeability (Tellez et al., 2014, 2015; Latorre et al., 2015). This indicates that rye can be used to induce nutritional deficiencies in chickens similar to those nutritional deficiencies in humans. There are also desirable features of a chicken model compared to a murine model including, their fast growth rates which allows for a reduction in trial time and similarity in hepatic lipogenesis between chickens and humans (Nafikov and Beitz, 2007). The development of an appropriate animal model would allow screening for various interventions and therapeutics in an attempt to ameliorate or reduce stunting. Also it appears that previous interventions used in an attempt to reduce stunting have be variable (Blanton et al., 2016; Mbuya and Humphrey, 2016). Access to various animal models may reveal new information attempt to reduce stunting.

There were three primary objectives for this dissertation. The first was to use published literature to review childhood stunting and the potential use of a chicken model to study childhood malnutrition. The second objective was to optimize the use of FITC-D to assess intestinal permeability in chickens. The third objective was to assess how three different genetic lines of chickens respond to four different dietary treatments that induce malnutrition during an early or late phase of growth

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Assessment of a Nutritional Rehabilitation Model in two modern broilers and their Jungle fowl ancestor

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Abstract

Inclusion of rye in poultry diets induces a nutritional deficit that leads to increased bacterial translocation, intestinal viscosity, and decreased bone mineralization. However, it is unclear the effect of diet on developmental stage or genetic strain. Therefore, the objective of this chapter is to evaluate the effects of a well establish rye model diet during either the early or late phase of development on performance, bone mineralization, and morphometric analysis. Furthermore intestinal integrity evaluated by liver bacterial translocation, leakage of FITC-d, and gene expression of tight junctions across 3 diverse genetic backgrounds: Modern 2015 (Cobb 500) broiler chicken, 1995 Cobb broiler chicken, and the Giant Jungle Fowl are also discussed.

Keywords: Nutritional rehabilitation, chicken lines, compensatory growth, bone mineralization, morphometric analysis, intestinal integrity

Introduction

Multiple metrics of growth are utilized when determining a child's nutritional status (Victora et al., 2010). Both height and weight relative to age are essential benchmarks when monitoring growth because these growth metrics exhibit similar trends across human development (Victora et al., 2010). Stunting is defined as low height/length for a child's age and is often reflected in poor linear growth (Victora et al., 2010; Prendergast and Humphrey, 2014), while wasting is low weight for length/height (Victora et al., 2010). It should be noted that before the age of 2, a child's height is measured as length. The World Health Organization (WHO) defines stunting as two or more standard deviations (SD) below the standard height for children at that particular age often referred to as Z score. The typical growth pattern for stunted children is a sharp decline in height/length from birth until 24 months of or until child reached 1.5-2SD below the median and then plateaued till 59 months (Victora et al., 2010). Globally, stunting, underweight or wasting are major contributors to morbidity and mortality in children (Prendergast and Humphrey, 2014). A report from 2000 indicated that globally stunting has decreased by 14% since the 1980's, however the reduction in stunting was unevenly distributed (De Onis et al., 2012). A projected trend for 2020 expect a decrease in the rate of stunting in Asia from 100 million to 68 million, while the number of stunted children in Africa, is expected to increase from 60 million to 64 million as the population increases (De Onis et al., 2012; Prendergast and Humphrey, 2014) . Globally, 26% of children under the age of five have a -Z score 2 standard deviations below the average, indicating stunting (Mbuya and Humphrey, 2016). Childhood stunting increase risk of mortality from an infectious disease reduces cognitive ability and lowers adult learning (Mbuya and Humphrey, 2016). Undernutrition in infants 6 months of age or younger is often attributed to low birth weights and breastfeeding patterns (Victora et al., 2010). The most vulnerable time for childhood stunting is

from conception until 24 months (De Onis et al., 2012). The long-term effects of stunting, like cognition, executive function and school attainment are most vulnerable to children during the first two years of life (Black et al., 2017). Stunting that occurs after this time is less correlated with the long-term effects of stunting (Black et al., 2017). Therefore the first two years of life is the time when interventions are more effective (Victora et al., 2010). Therefore, it is evident that prenatal and early life interventions are required to ensure proper growth.

It has been estimated that 43% (250 million) of children under the age 5 subjected to poverty and stunting will not reach their development potential (Grantham-McGregor et al., 2007; Black et al., 2017). A growing body of evidence documents that healthy children tend to become healthy and wealthy adults (Almond and Currie, 2011; Currie and Almond, 2011) and there is a positive correlation between higher birth weights and social, economic, and cognitive outcomes (Currie and Almond, 2011). For example, anthropometric markers such as birth weight and child height are related to future schooling, employment, earnings, family formation, and health (Currie and Vogl, 2013). Low to middle income countries has a higher risk of children not reaching their developmental potential due to poverty and stunting (Black et al., 2017). Exposure to poverty early in life affect the individual's health and wellbeing as an adult which can lead to 19.8% lower income than those individuals not exposed to stunted (Black et al., 2017). Low socioeconomic status in early childhood has been associated with smaller hippocampal gray matter, which has been associated with low cognition, academic and behavioral performance (Luby et al., 2013). Early life stressors have long-term effects that reach into adulthood where there are low task related activation of brain regions supporting language, cognition, memory and emotional reactivity (Pechtel and Pizzagalli, 2011; Council and others, 2015). Interventions to annihilate poverty have been shown to improve wage earning, intelligence, better health biomarkers, reduction violence,

depression and social inhibition (Black et al., 2017). The negative effects of early childhood stunting can also be attenuated when the child receives nurturing care (Black et al., 2017). Positive home environments had longer effects on cognition where children were more susceptible to their environment for up to 63 months (Black et al., 2017). Romanian children placed in foster care had a better cortisol response than those children who remained in an institution. The cortisol response may be the link between cognition and early childhood stressors (Black et al., 2017). Poverty often leads to exposure to multiple physical and psychological stressors, which can affect the child's physiological response as well as inhibit self-regulation and stress management (Black et al., 2017). For the first two years of life, macronutrient supplementation is for the increase intellectual development (Black et al., 2017). Nutrients promote healthy brain development (Black et al., 2017).Therefore it is evident that reducing the incidence of stunting can have positive long-term effects on the health and the economy and may be able break the cycle of poverty. The purpose of this review is to evaluate the etiology of stunting and present chickens as a viable model to study stunting in children.

Reduction in Stunting Case Studies

In Northeastern Brazil, there was a large decrease in the incidence of stunting in children from 33.9% in 1986 to 5.9% in 2006 (Lima et al., 2010). There were four primary reasons as to why this occurred was: low-income families had higher purchasing power, improved education of women with children, expanded public water and sewage systems and access to basic health care (Lima et al., 2010). Therefore, it is evident that improving sanitation, health care and education can reduce the stunting in children. There was also an increased employment, higher minimum wage income transfer program which resulted in a higher economic growth reducing the poverty

and balancing income distribution (Lima et al., 2010). Modest increases in the sewage network increased basic sanitation. Also, the average number of children per woman decreased from 5.2 in 1986 to 1.75 in 2006. The reduction in stunting also coincides with a family health program that was implemented nationwide promoting equal access to healthcare as well as preventive and health education (Lima et al., 2010).

The Mexican government implemented a food aid program in an attempt to reduce the incidence of stunting, however the program was unsuccessful (Rivera, 2009). This was due to low-income areas, that the program was intended for, did not have access to the resources. In addition, the program was not targeting children under the age of two, which is the age group most vulnerable to stunting. From there the government modified the program to distribute micronutrient-fortified food to meet the nutritional needs of infants 6 to 23 months of age in low-income areas. Two years after the modified food aid program was implemented, there was a significant reduction in the incidence of stunting in the poorest areas of Mexico but no effect on higher socioeconomic levels or older children than 6 months of age (Rivera, 2009).

Etiology of Stunting

The top five predictors of childhood stunting in India were maternal stature and weight, maternal education, household incomes and diversity in the diet (Corsi et al., 2016). Macro and micronutrient deficiencies also play a role in stunting. Semba et al. (2016) found that stunted children in rural Malawi have a low circulating level of nine essential amino acids and lower levels of sphingomyelins. In addition, certain phosphatidylcholines were in lower concentration in the serum suggesting that stunted children may also be deficient in choline (Semba et al., 2016).

Phosphatidylcholine and sphingomyelin play a major role in chondrogenesis, which can determine linear growth in bones (Semba et al., 2016). This study suggests that the inefficiency of micronutrient and lipid supplementation on stunting may be due to the deficiency in essential amino acids (Semba et al., 2016). There is also a direct relationship between systemic inflammation, growth hormone signaling and linear growth (DeBoer et al., 2017). Blocking tumor necrosis factors-alpha, via antibodies, reverse the GH signaling suggesting an interaction between GH and inflammation (Difedele et al., 2005). Systemic inflammation has been linked with higher level of GH, and lower levels of IGF-1 and IGF binding protein-3 (IGFBP-3) systemically and in the liver and lower linear growth (DeBoer et al., 2017). The higher systemic levels of inflammation are likely caused by the recurrent infections in children subjected to poverty (DeBoer et al., 2017). HsCRP (high sensitivity C-reactive protein), has been utilized as a biomarker of mild inflammation during viral or bacterial infections (DeBoer et al., 2017). Higher levels of serum hsCRP, was correlated with higher systemic and hepatic GH and lower levels of IGF-1 and IGFBP-3 (DeBoer et al., 2017). Higher GH and lower IGFBP-3 were associated with short stature and states of undernutrition. This data suggests that both diet and environmental pathogen exposure can have direct effects on growth in children.

Inflammation is the endpoint of stress, regardless of its origin or nature (biological, environmental, nutritional, physical, chemical or mental). Stress and inflammation are innate responses in living organisms involving hormones, immune cells and molecular mediators that are essential mechanisms for the survival and the healing process in all forms of life. However, if the system does not shut down, the consequence of chronic inflammation is devastator for the biology of the organisms and has been recognized as the “secret killer”. The interactions between diet ingredients, gut microbiome, nervous system, immune system and endocrine system play key roles in

metabolic and gastrointestinal disorders, diabetes, cancers, autoimmune diseases, malnutrition, obesity, cardiovascular and muscle function, and even neurological diseases.

A longitudinal study was conducted on Malawian twins from age 0 to 3 in rural communities to evaluate the effect of genetics on child malnutrition (Reyes et al., 2015). Between sets of twin pairs, there was a high rate of discordance in the effect of severe and moderate malnutrition (Reyes et al., 2015). In addition, nutrition alone was not an effective treatment for stunting, as feeding interventions only improved growth by 30%, suggesting that stunting is a multifactorial disease (Mbuya and Humphrey, 2016). Disease is another factor to consider when determining the etiology of stunting. Infections can affect nutrient absorption, which can lead to undernutrition and stunting. However, it should be noted that there is not a strong correlation between growth, diarrhea and disease (Mbuya and Humphrey, 2016). It has been theorized that unsanitary living environments lead to asymptomatic but chronic intestinal injury which results in immune stimulation and poor growth (Mbuya and Humphrey, 2016). An effective intervention in reducing the incidence of stunting focuses on three core issues water, sanitation and hygiene (WASH). Another factor that contributes to stunting is environmental enteropathy dysfunction (EED) (Blanton et al., 2016; Mbuya and Humphrey, 2016). EED is a subclinical disease of the small intestine characterized by villous atrophy, crypt elongation, infiltration CD8+ T-cells in the lamina propria and increase intestinal permeability associated with intestinal inflammation (Blanton et al., 2016; Mbuya and Humphrey, 2016). As well, there is an inverse relationship between enteric inflammation and linear growth and vaccine efficacy (Blanton et al., 2016). EED is prevalent in low-income countries with poor sanitation and high environmental loads of enteropathogenic bacteria, and is often associated with the pathogenesis of malnutrition (Blanton et al., 2016). The effects of EED are cyclic further proliferating growth faltering. The cycle starts with damage to the intestinal

morphology causing a loss of barrier function, which triggers hyperstimulation of the immune system (Blanton et al., 2016; Mbuya and Humphrey, 2016). This perpetuates the loss of barrier function and a reduction in absorptive function and secretion of digestive enzymes causing poor digestion and malabsorption (Blanton et al., 2016; Mbuya and Humphrey, 2016). The etiology of EED is unclear but the continuous exposure to pathogenic bacteria and their enterotoxins cause villous atrophy that correlates with crypt hyperplasia (Mbuya and Humphrey, 2016). This causes villous blunting which reduces absorption capacity and fewer secretions. Also, the high intestinal pathogenic load causes hyperstimulation of enteric T-cells which contribute to the crypt hyperplasia (Mbuya and Humphrey, 2016). Therefore, both the host's immune system and the pathogenic bacteria are causing mucosal damage. The hyper stimulation of the cell-mediated immune response is thought to occur due to the high concentration of fecal microorganisms but may also be caused by severe nutritional deficiency, HIV, or mycotoxin exposure (Mbuya and Humphrey, 2016). Certain pathogens and/or endotoxins can disrupt the intestinal barrier via tight junction or by activating pro-inflammatory immune mediators. Chronic pathogen exposure causes chronic immune activation. Intravenous infusion of endotoxin administered to healthy humans increase gut permeability (O'Dwyer et al., 1988). There is a correlation between intestinal permeability and stunted growth, where 55% of linear growth faltering occurred while Gambian infants had impaired intestinal permeability (Campbell et al., 2003). There are thought to be three primary reasons growth is impaired during EED. First, the hyperstimulation of the immune response it metabolically expensive (Campbell et al., 2003). Second, proinflammatory cytokines can act to reduce growth related hormones impeding growth (Campbell et al., 2003). Lastly, proinflammatory cytokines can attenuate bone remodeling causing more permanent stunting (Campbell et al., 2003). Regardless if the child had diarrhea, pathogenic bacteria was found in the

stool of children with EED under the age of 60 months in both sub-Saharan Africa and south Asia (Kotloff et al., 2013). Suggesting that there is subclinical inflammation caused by EED is a major contributor of stunting (Kotloff et al., 2013). Malabsorption may also play a role if the severity of EED causes a high abundance of nutrient loss in the stool (Kotloff et al., 2013). It is evident that regardless of etiology of EED, the disease is dependent on the environment. In areas where the incidence of EED is high, newborn do not have the intestinal histopathology associated with EED and removing individuals with EED from the environment allow them to restore normal intestinal morphology and barrier function (Haghighi et al., 1997). However, it should be noted that recovery of this disorder is relatively slow and individual that who presumably had the condition longer take longer to recover (Mbuya and Humphrey, 2016). Bangladeshi children living in environmentally clean households had less severe EED and higher HAZ than children from contaminated households (Lin et al., 2013).

There are limited efficacious treatments for EED; antibiotics have been shown to have modest improvements in growth (Trehan et al., 2009; Blanton et al., 2016). Improving micronutrient status via supplementation did not affect linear growth (Blanton et al., 2016). Energy dense, micronutrient fortified ready to use therapeutic food can accelerate short-term weight gain affecting metabolism by switching from fatty acid oxidation to amino acid oxidation, which increases fat deposition and weight gain (Blanton et al., 2016). There is a lack of information on the optimal time and duration of the nutritional intervention (Blanton et al., 2016).

It is evident that stunting and malnutrition is a multifactorial issue and that the microbiota plays a role in the mediating nutrition and pathogenesis of disease. It has been suggested that in order to determine the role of the microbiota there needs to be a benchmark set to determine significant changes (Blanton et al., 2016). Microbiota for age Z is currently being defined as the degree of

deviation of an unhealthy individual microbiota age from a reference cohort of a chronological age matching child with normal growth. This data revealed that Bangladeshi and Malawian children had “immature” gut microbiota which a similar bacterial profile of child that is younger (Smith et al., 2013; Subramanian et al., 2014). They hypothesized that microbiota maturation is functionally linked to the growth rate of the host (Smith et al., 2013; Subramanian et al., 2014). Breast milk contains a lactose core and linked glucose, galactose, N-Acetyl galactosamine, fructose, and or sialic acid residues (Blanton et al., 2016). These carbohydrate sources have prebiotic actions to promote colonization of bifidobacterial taxa. Bifidobacterium have multiple benefits to the host including improved vaccine response, enhanced gut barrier and protection from enteric infection (Blanton et al., 2016). Gnotobiotic mice that were colonized with bacteria isolated from stunted infants in Malawi were supplemented with sialylated bovine milk oligosaccharide (BMO) or fructo-oligosaccharides given a micro and macronutrients deficient diets (Blanton et al., 2016). They found that mice on the BMO diet exhibited growth increases that were dependent on the microbiota (Blanton et al., 2016). Therefore it is evident that microbiota play a key role in modulating growth and EED.

Animal agriculture has shown that low levels of antibiotics can reduce the number of pathogenic bacteria (Mbuya and Humphrey, 2016). It is evident from animal trials that growth and intestinal morphology can be improved when the animals are placed in an environment with a low bacterial load and environmental immunogens. Research conducted in Bangladesh found that household with lower levels of parasites and less severe EED had better growth than those in less hygienic environments (Lin et al., 2013). This suggests that improved household sanitation and hygiene in areas with high parasitic loads can improved the severity of EED. However, the degree of sanitation matters as highly contaminated areas with little access to clean water found that hand

washing was not able to reduce the levels of subclinical mucosal damage and immune stimulation (Langford et al., 2011). Infant exploratory behaviors can lead to increased ingestion of pathogenic bacteria (Marquis et al., 1990; Ngure et al., 2013). Therefore, it is evident that still much is unknown about EED. Things to investigate further, the etiology of EED regarding the microbial species, required dose to cause disease, the role of maternal EED in utero and after birth; potential preventative including antibiotics, probiotics and anti-inflammatory (Mbuya and Humphrey, 2016)

Compensatory Gain

Compensatory growth, also known as catch-up growth or compensatory gain, is an accelerated growth of an organism following a period of slowed development, particularly because of nutrient deprivation. Growth may be measured with respect to weight, length or height in humans (Faruque et al., 2008; Prabhakaran et al., 2008a). In some instances, body weights of animals under feed restriction will catch-up to control animals with *ad libitum* feed intake (Wilson and Osbourn, 1960; Yair and Uni, 2011). In fact, high compensatory growth rates in feed restriction animals result in overcompensation due to excessive fat deposition and animals recover to normal weight without additional time (Zubair and Leeson, 1996; Zhan et al., 2007). Nevertheless, when the nutrient restriction is severe, the growth period must be extended to reach the normal weight, but if the nutrient restriction is severe enough, permanent stunted growth may occur (Pelletier et al., 1995). Compensatory growth has been reported in metazoans, plants, fungi and even prokaryotes (Zubair and Leeson, 1996; Mikola and Setälä, 1998; Bretherton et al., 2006; Turano et al., 2008). Although the exact biological mechanisms for compensatory growth are poorly understood, it is clear that in some animals the endocrine system is involved (Turano et al., 2008). During the first stages of

starvation, there is a reduction of basal metabolism (Rosebrough and McMurtry, 1993). The intestinal tract is the first organ to be reduced in both weight and activity (Jin et al., 1994; Agyekum et al., 2012). Then, as feeding, is normalized, dietary protein and energy supports intestinal growth, followed by muscular tissue and at the end adipose tissue (Yamauchi et al., 2010). Some of the factors that affect compensatory growth include (Rosebrough and McMurtry, 1993) composition of the restricted diet; severity of undernutrition; duration of the period of undernutrition; age; genotype; and gender among others (Zulkifli et al., 2001, 2009; Prabhakaran et al., 2008b). An epidemiological study determined that 56% of childhood mortality (aged 6 to 59 months) was attributed to malnutrition potentiating effects, and 83% of these was attributed to mild-to-moderate as opposed to severe malnutrition (Pelletier et al., 1995). This and other studies propose that malnutrition plays a major role on child mortality and suggest that strategies involving only the treatment of the severely malnourished is not enough to reduce the negative impacts of malnutrition (Barendregt et al., 2008; Smith et al., 2013; Ahmed et al., 2014). Furthermore, malnutrition remains the major focus of nutritional intervention efforts, especially because dietary deprivation during early life can also have adverse effects on brain anatomy, physiology, biochemistry, and may even lead to permanent brain damage (de Souza et al., 2011). When diarrhea was followed by diarrhea free periods children exhibited compensatory gain and re-entered the growth trajectory (Mbuya and Humphrey, 2016).

Chickens as a model for research in human

Using appropriate animal models is essential when studying human health (Baker, 2008). Chicken have been an important experimental model in biology for more than 2 thousand years having led to many central discoveries (Tickle, 2004; Stern, 2005; Burt, 2007). However, with the latest

advances in genetics and nutrition technologies, chickens have attained a superb model organism status (Pourquié, 2004; Stern, 2005). Hence, chickens are the system of choice for many vertebrate biologists, especially in the field of human sciences, who are interested in gene function (Tickle, 2004; Cogburn et al., 2007), as well as nutrition (Stern, 2005). Avian species have also been utilized as model organisms to neuron and brain development. Bock and Braun, (1998) found that chicks imprinted by a surrogate mother with auditory tones stimulated synaptic reorganization correlated with learning and memory processes. This synaptic proliferation and elimination has been observed during early and late childhood and are associated with emotional experience and learning (Bock and Braun, 1998). Typically pigs are used as a model to study human nutrition because rodents' vastly different nutrient requirements and nutrient-nutrient interaction, as well as they are coprophagic and utilize different feeding strategies (Roura et al., 2016). Roura et al., (2016) reported that the mechanisms of intestinal permeability and intestinal immunity systems are well conserved across species, however pigs often make an excellent model for humans due to their similarity in gastrointestinal anatomy. Although chickens have a shorter intestinal tract when compared to humans (Sturkie, 2012), there are many reasons as to why chicken are an appropriate model to study human nutrition. The first being the liver is the primary site of lipogenesis in both chicks and humans (Nafikov and Beitz, 2007). In addition, both neonatal chicks and humans are able to efficiently utilize sucrose as an energy source. (Baker, 1997). In both chickens and humans, iron is primarily absorbed in the duodenum (Sturkie, 2012).

Regardless of species, the intestinal epithelium constitutes the largest immune organ and most important barrier against external environmental agents and has two critical functions: to prevent the entry of harmful intraluminal microorganisms, antigens, and toxins, and to enable the selective translocation of dietary nutrients and electrolytes into circulation (Salminen et al., 2006;Salzman,

2011; Elson and Cong, 2012). One of the basic properties of gut-associated lymphoid tissue (GALT) is oral tolerance (unresponsiveness) to harmless components of microbiota and diet (Kau et al., 2011). Inappropriate immunological reactions against food compounds, such as lactose or gluten, can lead to the breakdown of oral tolerance and the development of intestinal immune disorders (Stepniak et al., 2006) . For example, Celiac disease (CD) is a chronic immune-mediated enteropathy of the small intestine that is triggered by dietary wheat gluten, or related rye and barley proteins in genetically susceptible individuals (Williamson and Marsh, 2002). The clinical and pathological spectrum of CD is heterogeneous and there is no current rodent model that reproduces all aspects of human celiac disease (Schuppan et al., 2009; Kupfer and Jabri, 2012). Patients display intestinal barrier dysfunction and altered tight junction protein expression allowing abnormal penetration of gluten-related peptides and enteric microorganisms, which could stimulate any subsequent immune response (Ströhle et al., 2013). Clinical presentation of CD can vary from a classical malabsorption syndrome to more subtle atypical gastrointestinal manifestations (similar to irritable bowel syndrome) or extra intestinal presentations (infertility, osteoporosis, and iron-deficiency anemia) (Bianchi and Bardella, 2008; Assimakopoulos et al., 2011; Pelkowski and Viera, 2014). Likewise, the composition of the diet, also has a tremendous impact in digestibility and gut health of chickens (Dunsford et al., 1989; Hrncir et al., 2008; Maslowski and Mackay, 2010). A specific example is rye-based diets versus traditional corn-based diets, where different cereals are used as the principal source of energy. The inclusion of rye in poultry diets has been fraught with problems, principally related to the production of sticky droppings, malabsorption syndrome, elevated feed conversion and intestinal bacterial overgrowth (Campbell et al., 1983; Bedford and Schulze, 1998; Shirzadi et al., 2010). The endosperm cell wall of rye and wheat is comprised mainly of highly branched arabinoxylans, which increase the

viscosity of the digesta (Bedford and Classen, 1993). Elevated viscosity reduces digestibility and performance by interfering with the movement of particles and solutes across the intestinal lumen and causing dysbacteriosis and severe gut inflammation (Annett et al., 2002; Shirzadi et al., 2010). Hence, our laboratory made a breakthrough by developing a human-relevant nutritional chicken model to induce intestinal inflammation (Tellez et al., 2014). Although pigs are the model organism for conducting human nutritional research it appears that poultry have a more severe reaction to rye based diets. It has been hypothesized that pigs are able to digest non-starch polysaccharides (NSP) better than poultry due to the high volume of the large intestine allowing for more fermentation and longer transit time of the digesta (Choct and Cadogan, 2001). It also should be noted that chicks are able to double their starting body weight in 3 days, where it takes pigs 20 days and in children it 5-6months (Baker, 2008). Therefore, from a practical standpoint, more trials can be completed in a shorter amount of time.

Chicken and Rye

Starch and NSP's are a primary carbohydrate sources in plants (Bederska-\Lojewska et al., 2017). Cellulose arabinoxylans and β-glucans are the primary NSPs and require microbial digestion to be utilized by monogastric animals (Bederska-Lojewska et al., 2017). Arabinoxylans are the primary component of the thin lignified cell wall of the endosperm (Cozannet et al., 2017) and insoluble arabinose and endogenous enzymes do not efficiently degrade xylose residues. NSP's can be further classified by their water solubility, which is dependent on the chemical structure of these sugars (Bederska-Lojewska et al., 2017). The greater the solubility of the polysaccharide, the more viscous the digesta which reduces it nutrient availability. Rye is an economical raw feed material to produce as it is tolerant to low temperature and drought, irregular soil pH and requires less

chemical treatments (Bederska-Lojewska et al., 2017). However on a dry matter basis, rye grain contains 9.7% soluble NSPs likely in the form of arabinoxylans. Hybrid rye variants have been bred to reduce the amount of anti-nutritional factors allowing rye to be added to poultry diets at a higher inclusion rate (Bederska-Lojewska et al., 2017).

Soluble NSPs have beneficial effects on human health by lowering blood sugar levels, facilitating regular bowel movements and reducing risk of heart disease and other metabolic syndromes (Dhingra et al., 2012). Wild avian species consuming NSPs as whole grains, which prolongs digestion time in the crop and allows for more microbial digestion, eliminating some of the anti-nutritional factors (Bederska-Lojewska et al., 2017). In modern agricultural animals, diets containing excessive amounts of NSPs negatively affect health and perpetuates a state of disease (Bederska-Lojewska et al., 2017). It is evident from the literature that broiler chickens consuming diets high in NSPs increases ileal viscosity which leads to less interaction of endogenous enzymes and nutrients and reduces nutrient digestion (Bederska-Lojewska et al., 2017). Lower digestibility, results in less energy available for growth, which reduces BW and increases feed consumption that increase production costs (Bederska-Lojewska et al., 2017). It has also been reported that chickens consuming diets high in NSPs have increased feed intake in an attempt to maintain nutrient intake which increased the transit rate and increased intestinal viscosity even further (Bedford, 2006). Broilers consuming wheat-based diets had significantly higher gut viscosity, reducing AME and depressing growth and feed conversion efficiency (Choct et al., 1996). Broilers and turkey poults consuming rye as the primary carbohydrate source had increased digesta viscosity, increased intestinal permeability, reduction in bone strength and mineralization and changes in microbial composition (Tellez et al., 2014, 2015; Latorre et al., 2015). The anti-nutritive effects of rye were attenuated when the diet was supplemented with *Bacillus* based direct fed microbial (Latorre et

al., 2015). Inclusion of 5 and 10% rye from d14 to 28d decreased performance and litter quality and increased gene expression of cellular growth and differentiation in cell survival processes (van Krimpen et al., 2017). Rye also upregulated complement and coagulation signaling pathway which is characteristically upregulated to eliminate infections (van Krimpen et al., 2017). Laying hens fed a diet containing rye, had a reduction in egg production, feed conversion efficiency and eggshell cleanliness (Lázaro et al., 2003). The anti-nutritive effects of rye in laying hens could be improved when the diet was supplemented with a NSP degrading enzyme complex (Lázaro et al., 2003). The higher digesta viscosity of soluble NSP diets also increase litter moisture, which can increase the incidence of footpad dermatitis (Bederska-\Lojewska et al., 2017).

There is a negative correlation between the digestibility of fat and dietary fiber inclusion (Cozannet et al., 2017). Saturated fats sources such as tallow require larger intestinal surface area to be absorbed and have higher melting temperature affecting digestion compared to unsaturated fatty acids (Dänicke et al., 1997). Therefore, dietary fat properties and fiber inclusion rate affect fat digestion and absorption. To increase the solubilization of long chain saturated fatty acids bile salts and unsaturated fatty acids are required in the micelle (Dänicke et al., 1997). The increased digesta viscosity associated with high NSP diets, reduces fat digestibility by interfering with emulsification and subsequent absorption (Dänicke et al., 1997). The reduction in fat absorption of chicks consuming diets high in NSP also puts chicks at risk for fat-soluble vitamin deficiencies. It has been previously observed that hepatic vitamin E levels were significantly lower in rye fed birds (Danicke et al., 1999). Higher viscosity also increases gastric passage rate, which can increase the possibility of pathogen proliferation. Higher potential pathogenic load within the lumen of the intestinal tract can increase risk of bacterial translocation stimulating the inflammatory response that increases intestinal leakage, and leads to higher amounts of bacterial

translocation (Bederska-\Lojewska et al., 2017). Rye fed chicks also had a higher abundance of coliforms in the small intestine (Latorre et al., 2015). The higher abundance of coliforms in high NSP diets was also observed in an *in vitro* system (Krause et al., 2008). Adding silage, rye and chicken manure to a biogas reactor led to a high abundance of *Clostridia*, which play a vital role in the digestion of polysaccharides and oligosaccharides (Krause et al., 2008). There have been varied observations on the effect of soluble fibers on microbial population (Bederska-\Lojewska et al., 2017). Diets containing 10% rye decreased population of commensal bacteria such as *Lactobacillus* (van Krimpen et al., 2017). Certain populations of commensal bacteria can utilize resistant starches, NSPs, oligosaccharides or proteins to produce short chain fatty acids (SCFA) which can be used as an energy source by the animal (Bederska-\Lojewska et al., 2017). Particular types of SCFA are able to cross the lipid membrane of prokaryotes where they dissociate in the cytoplasmic, destroying the bacterial cells (Bederska-\Lojewska et al., 2017). SCFA also reduce the luminal pH, which can limit pathogen proliferation (Bederska-\Lojewska et al., 2017). Soluble fiber has also been reported to prevent the adherence of certain pathogenic bacteria to epithelial cells (Roberts et al., 2013). (Mathlouthi et al., 2002) reported that wheat and barley consumption, increases bacteria in the ceca – both commensal (*Lactobacillus* strains) and pathogenic (*E. coli*). Non-ruminant enzymes are unable to degrade arabinoxylans which enter the colon relatively intact where they stimulate growth of residing bacteria such as *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus*, and *Eubacterium* (Rivière et al., 2014). Chickens consuming a wheat/rye diets resulted in a higher abundance of mucosa associated bacteria especially enterobacteria and enterococci (Hübener et al., 2002). This indicates that the higher digesta viscosity associated with a rye based diet, results in an increased in the bacterial activity in the small intestine (Hübener et al., 2002). Also, NSP's containing diets that were not supplemented

with enzymes, had significantly more ileal volatile fatty acids, which indicates higher bacterial fermentation (Choct and Annison, 1992). Furthermore, preliminary microbiome analysis from our laboratory found drastic differences in cecal microbiome profiles between chicks consuming rye and corn-based diets (Figure 1). Rye fed chicks had a higher abundance of beneficial bacteria such as *Lactobacillus and Bifidobacteria* but also a higher abundance of potentially pathogenic bacteria including *Clostridium* and *Proteus*, indicating dysbacteriosis. Corn fed chicks had a higher abundance of SCFA producing bacteria such as *Faecalibacterium*, *Dorea*, *Oscillospira* and *Blautia* which may be more representative of a "healthy" microbiota. Soluble NSP's have been reported to improve the development of the intestinal mucosa by increasing villus height and crypt depth in broilers consuming a diet containing 10% rye (van Krimpen et al., 2017). Insoluble fibers have also shown to improve intestinal morphology by increasing absorptive surface area (Sarikhan et al., 2010). Broilers fed a barley based diet had changes in intestinal morphology compared to those birds fed corn-soy where there were shorter and thicker and atrophied villi, and increased goblet cell size (Viveros et al., 1994).

High NSP diets supplemented with enzymes have shorter passage time by decreasing digesta viscosity (Danicke et al., 1999). Supplementation with starch degrading enzymes can ameliorate some of these negative side effects while there was no observed effect of antibiotics supplementation on performance parameters (Choct et al., 1996). Addition of a multi carbohydrase enzyme complex containing both xyalanses and arabinofuranosidases improve digestibility of diets containing various amount of different NSPs (Cozannet et al., 2017). The higher apparent metabolizable energy of diets was attributed to the starch crude protein and crude fiber but the NSPs did not increase energy availability (Cozannet et al., 2017). The proposed mechanism of action of this enzyme complex is that the carbohydrases allow for improved endogenous enzyme

and substrate interaction, allowing for improved digestibility (Cozannet et al., 2017). Similar improvements in broiler performance were observed when the cereal grain was soaked in water where there was a decrease in digest viscosity, increased growth parameters, increased villi height and reduced muscularis thickness and crypt depth proliferation, and increase VFA concentration in the ceca (Yasar and Forbes, 2000). (Pettersson et al., 1991)also found that steam-pelleting diets containing barley, wheat and rye had increased digestibility in broiler chickens. Young chicks are less tolerant to high NSP's diets because the higher digesta viscosity limits peristalsis which prevents the maintenance of digesta flow rate (Bedford, 2006). The inability of nutrients to move through the intestine rapidly prevents the absorption of nutrients to meet energy requirements for maintenance (Bedford, 2006). Antibiotics growth promoters are thought to improve performance via modulation of the intestinal microbiome (Pan and Yu, 2014). Previous research has suggested that antibiotics supplemented with rye based diets attenuated some of these effects by eliminating the ability of the microbiota the ferment soluble NSP (MacAuliffe and McGinnis, 1971). However, Choct and Annison (1992) did not observe any improvement in digestion and growth when antibiotics were added to the feed, which may be due the antibiotic used in this study.

The aim of future studies is to evaluate a nutritional rehabilitation in chickens to determine if they are an appropriate model to study interventions in childhood malnutrition. To the best of our knowledge there is limited information on if nutrition alone can facilitate intestinal recovery after the consumption of a rye based diet in chickens. The model utilized rye and corn to evoke early or late phase malnutrition in three different genetic lines of chickens. To study early phase malnutrition chicks were fed rye for the first ten days and then switched to a corn based diet. To study late phase malnutrition, chicks were fed a corn-based diet for the first ten days and then switched to rye-based diet. The two control groups were maintained on a rye or corn-based diet

throughout the experiment. Preliminary results from our laboratory had comparable results to what has been reported in rye fed chicks. Figure 2 illustrates that rye fed Jungle fowl (B) and modern broilers (C) had feces pasted to their vents while the corn-fed birds had more normal fecal viscosity (A). As mentioned above this is likely due to the higher digesta viscosity caused by diets containing high amounts of NSP's. Figure 2 (D) also illustrate the drastic difference in body weight between modern broilers fed a corn based diet (red arrow) and those fed a rye based diet (green arrow). Litter quality was another qualitative observation made when chicks were fed high NSP diets. Figure 3A illustrate that modern broilers maintained on a rye based had higher littler moisture. Broilers in the early phase malnutrition group (Figure 3B) appeared to have comparable litter moisture to those broilers maintained on the control corn fed diet (Figure 3C). This indicates that the modern broilers in the early phase malnutrition groups were able to reduce digesta viscosity which reduced litter moisture. Although Jungle fowl had pasted feces (Figure 2), consumption of a rye-based diet appeared to have little effect on litter moisture (Figure 3D). There was observable difference in body weight, litter moisture and pasted feces in the modern broilers, however there are no obvious differences in histology of ileum consuming the various diets (Figure 4).

Conclusion

Almost half of children under the age 5 are living in impoverish conditions putting them at greater risk of becoming stunted. The short-term effects of childhood stunting increase risk of mortality from an infectious disease, and has long-term effects like reduces cognitive ability and lowers adult learning (Mbuya and Humphrey, 2016). It is evident from case studies that the incidence of stunting can be reduced when people had access to primary health care and education, increased sanitation, improve wealth distribution and access to food (Rivera, 2009; Lima et al., 2010). However, treatment opportunities tend to be time sensitive and most effective to reduce long-term

effects of stunting is if implemented within the first two years of life. Multiple factors contribute to the etiology of stunting, making it difficult to find a treatment. There has been limited treatment success by improving diet alone and high environmental load of enteropathogenic bacteria can affect nutritional statue and growth (Mbuya and Humphrey, 2016). The histopathology of EED includes villous atrophy, crypt elongation, increase intestinal permeability and intestinal inflammation (Blanton et al., 2016; Mbuya and Humphrey, 2016) which can be observed in stunted patients. As well, there is an inverse relationship between enteric inflammation and linear growth and vaccine efficacy (Blanton et al., 2016). However, treating EED with antibiotics has had limited success on improving growth (Trehan et al., 2009; Blanton et al., 2016). There is a lack of information on the optimal time and duration of the nutritional intervention (Blanton et al., 2016). Therefore, to determine effective ways to treatment stunting a viable animal model is essential. Avian species are a common animal model for human research especially in the field of gene function, nutrition, immunology and developmental biology. The physiological response of poultry to a rye based diet is like what is observed in patients with EED. The inclusion of rye in poultry diets has been fraught with problems, principally related to the production of sticky droppings, malabsorption syndrome, poor growth performance, increased intestinal permeability and intestinal bacterial overgrowth (Campbell et al., 1983; Bedford and Schulze, 1998; Shirzadi et al., 2010; Tellez et al., 2014, 2015). The similarities between poultry consuming a rye based diet and patients with EED and stunted children suggest that chickens would make a viable stunting model to determine potential interventions and treatments.

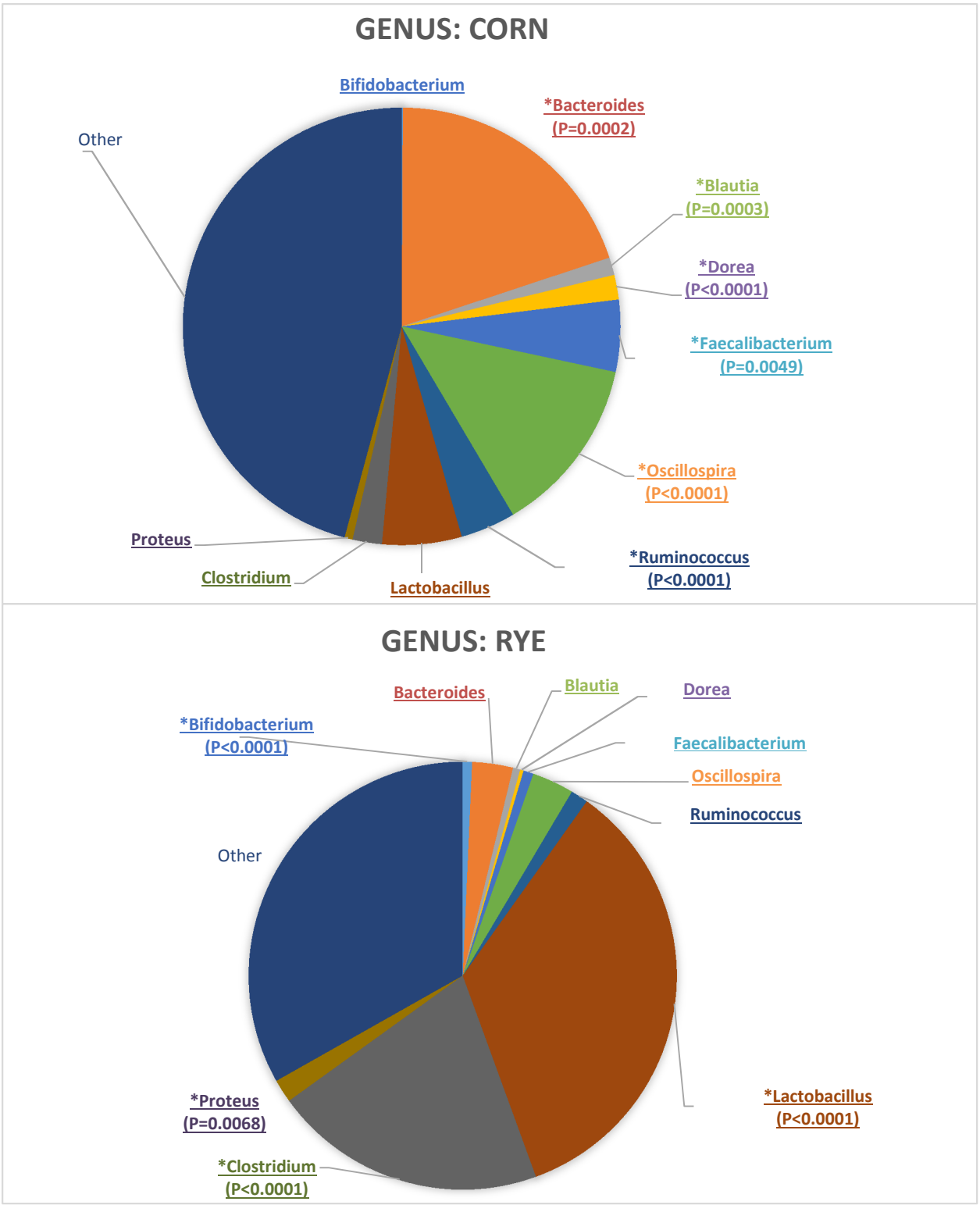

Figure 1: Cecal Microbiome of 10 day old broiler chicks consuming rye or corn based diets at the genus level**.** Data is expressed as relative abundance (%). *indicates significant differences in genera between chicks fed a corn and rye based diet.

Figure 2: Visually comparing fecal viscosity and body weight of rye and corn fed Jungle Fowl and Modern broilers. (A) Jungle fowl consuming a corn based diet had feces with relatively normal consistency (red arrow) and a relatively clean vent. (B) Jungle fowl consuming a rye based diet had feces pasted to the vent and diarrhea like consistency (red arrow). (C) Modern broilers consuming a rye based diet had diarrhea pasted to the vent. (D) Illustrates the size difference and overall appearance of between modern broiler chicks fed a corned based diet (red arrow) and a chick fed a rye based diet (green arrow)

Figure 3: Litter Quality and Behavior observation of chickens consuming a rye or corn based diets .(A) Modern broiler maintained on a rye based diet for 20 days of age had higher litter moisture and tended to huddle (red arrow). (B) Modern broiler maintained on a rye based diet from 0-10 days of age and then the diet was switched to a corn based diet from 10-20 days of age had a reduction in litter moisture. (C) Modern broiler maintained on a corn based diet for 20 days of age and appear to have similar litter moisture to those chicks consuming rye in the first phase of the experiment. (D) Jungle fowl maintained on a rye based diet exhibited diarrhea (seen in Figure 1), however little moisture did not appear to be affected by the diet as.

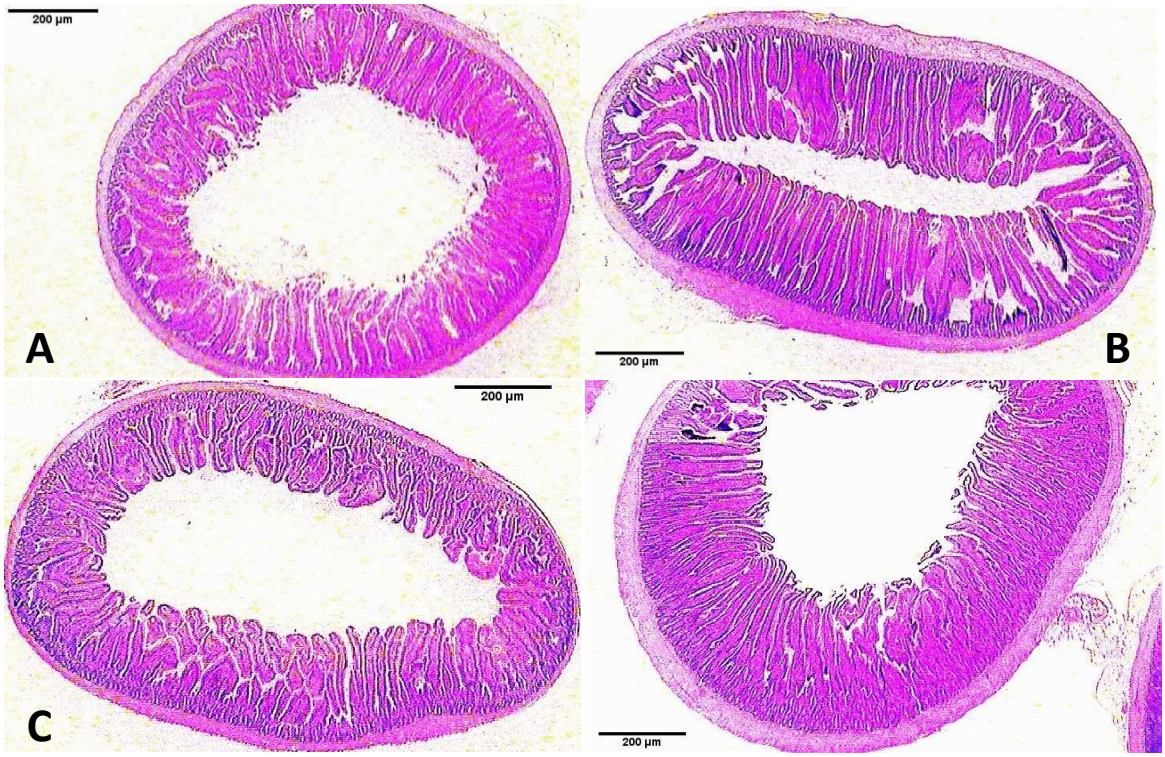

Figure 4: Histology of the Ileum in modern broilers on a corn or rye based diet program. The histology of the ileum of chicks maintained on different combinations of rye and corn based diet. (A) Modern broiler maintained on a corn based diet from hatch to 20 days of age. (B) Modern broiler maintained on a rye based diet from 0-10 days of age and then the diet was switched to a corn based diet from 10 to 20 days of age. (C) Modern broiler maintained on a rye based diet from hatch to 20 days of age. (D) Modern broiler maintained on a corn based diet from 0 to10 days of age and then the diet was switched to a rye based diet from 10 to 20 days of age.

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Optimizing fluorescein isothiocyanate dextran (FITC-d) measurement as a biomarker in a 24 h feed restriction model to induce gut permeability in broiler chickens

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Abstract

Fluorescein isothiocyanate dextran (FITC-d) is a 3-5kda marker used to measure tight junction permeability. We have previously shown that intestinal barrier function can be adversely affected by stress, poorly digested diets, or feed restriction, resulting in increased intestinal inflammation-associated permeability. Further optimization adjustments of the current FITC-d methodology are possible to enhance precision and efficacy of results in future studies. The objective of the present study was to optimize our current model to obtain a larger difference between control and treated groups, by optimizing the FITC-d measurement as a biomarker in a 24 h feed restriction (FR) model to induce gut permeability in broiler chickens. One *in vitro* and four *in vivo* independent experiments were conducted. The results of the present study suggest that by increasing the dose of FITC-d, (8.32 mg/kg *versus* 4.16 mg/kg); shortening the collection time of blood samples, (1 h *versus* 2.5 h); using a pool of non-FITC-d serum as a blank, compared to previously used PBS; adding a standard curve to set a limit of detection and modifying the software's optimal sensitivity value it was possible to obtain more consistent and reliable results.

Keywords: Enteric Inflammation, FITC-d, gut permeability, feed restriction, broiler chickens

Introduction

Intestinal epithelial cells are not only responsible for digestion, secretion and absorption but act as a physical barrier separating external environmental agents from the internal host environment. In addition to preventing the entry of harmful intraluminal microorganisms, antigens, and toxins, this barrier increases the bodies tolerance to nutrients, water and electrolytes (Salminen and Isolauri, 2006; Salzman, 2011; Elson and Cong, 2012). Microbes that live inside and/or on animals outnumber the animals' actual somatic and germ cells by an estimated 10-fold (Neish, 2009). Hence, any alterations in gut permeability are associated with bacterial translocation to the portal and/or systemic circulation leading to systemic bacterial infections (Ilan, 2012; Seki and Schnabl, 2012). Consequently, our laboratory has recently developed several models to induce intestinal inflammation in poultry. Those models include high non-starch polysaccharides diets (Tellez et al., 2014; Tellez et al., 2015); dexamethasone (Vicuña et al., 2015a); dextran sodium sulfate (DSS) (Kuttappan et al., 2015a; Menconi et al., 2015); and 24 h feed restriction (Vicuña et al., 2015b; Kuttappan et al., 2015b). In the above models, inflammation causes disruption of the epithelial tight junctions increasing bacterial translocation and leakage of serum Fluorescein isothiocyanate dextran (FITC-d). FITC-d is a large molecule (3 to 5 kDa) which under normal conditions is not able to cross the epithelial barrier (Yan et al., 2009). However, during intestinal inflammation the tight junctions are disrupted allowing the FITC-d molecule to enter circulation. Previous results from our laboratory have demonstrated that in poultry, chemically induced disruption of tight junctions with DSS (Kuttappan et al., 2015a) increases trans mucosal permeability as seen by elevated serum levels of FITC-d (Menconi et al., 2015). On the other hand, recently, we have shown that dietary inclusion of a *Bacillus*-based direct-fed microbial (DFM) ameliorated the adverse gut permeability inflammatory effects related to utilization of rye-based diets in turkeys

and in broilers chickens (Latorre et al., 2014; Latorre et al., 2015). We have previously shown that FITC-d can be used as a biomarker for intestinal barrier function (Tellez et al., 2014; Tellez et al., 2015; Kuttappan et al., 2015a; Kuttappan et al., 2015b; Menconi et al., 2015; Vicuña et al., 2015a; Vicuña et al., 2015b). However, further optimization adjustments of the current FITC-d methodology are possible to enhance precision and efficacy of results in future studies as can be observed in Table 5. The objective of the present study was to optimize our current FITC-d model to obtain a larger difference between control and treated groups, using our 24 h feed restriction (FR) model to induce gut permeability in broiler chickens.

Materials and Method

Fluorescein Isothiocyanate Dextran (FITC-d)

Fluorescein Isothiocyanate dextran (MW 3-5 KDa; Sigma Aldrich Co., St. Louis, MO) was used as a marker of paracellular transport and mucosal barrier dysfunction.

In Vitro Evaluation of different fluorescence gain using blank chicken sera from chickens without FITC-d

Unlike absorbance assays where the gain on the plate reader is fixed and not user changeable, fluorescence assays have varying concentration ranges and require the gain on the photomultiplier (PMT) to be adjusted. In this *in vitro* experiment, the following formula was used to predict the Relative Fluorescence Units (RFU) when changing the gain:

Estimate of RFU at new gain setting = (new PMT gain / old PMT gain)$^{7.3}$ * RFU at old PMT gain.

To determine if fluorescence changes with varying gain, blank chicken sera and 0.9% saline were compared. Non-FITC-d chicken sera was diluted 1:5 in 0.9% saline onto black 96-well fluorescent plates and measured from gain 40 to gain 80 with continuous increments of 10. Non-FITC-d sera

were also used to develop a standard curve adapted for every plate using 6 two-fold serial dilutions from the highest value 6400 ng/mL until it reaches 0 ng/mL (Table 1).

Experimental Animals

Four *in vivo* experiments were conducted to determine the optimal procedure for using FITC-d as a biomarker for intestinal permeability. In all trials, broiler chickens were obtained from a primary breeder company and all experiments were conducted in battery cages in a controlled age-appropriate environment. All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas.

Feed Restriction Model

In all experiments, intestinal permeability was induced using FR as previously published (Kuttappan et al., 2015b; Vicuña et al., 2015b). Chickens were randomly assigned to each experimental group and had unrestricted access to feed and water from 1 d to 10 d of age. Beginning at 10 d, chickens in control FITC-d groups, were allowed to continue with *ad libitum* access to feed, while chickens in FR FITC-d groups, were subjected to 24 h of FR. Concentration of FITC-d was given based on group body weight, therefore groups were weighed the day before FR began. At 11 d of age, chickens in all groups were given an appropriate dose of FITC-d by oral gavage for each experiment. After 1 h, or 2.5 h respectively, chickens were euthanized with CO_2 asphyxiation. Blood samples were collected from the femoral vein to quantify levels of FITC-d.

Serum Determination of FITC-d.

In all experiments, blood was centrifuged (1,000 x *g* for 15 min) to separate the serum from the red blood cells. FITC-d levels of diluted sera were measured at excitation wavelength of 485 nm

and emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., Vermont, USA). Fluorescence measurements were then compared to a standard curve with known FITC-d concentrations or non-FITC-d sera obtained from each independent experiment respectively to develop a standard curve as described in the *in vitro* methods.

Experimental designs

Experiment 1.

Comparing two dilution methods on serum FITC-d read at gain 70 in a 24 h feed restriction model

Eighty chickens were randomly assigned to one of 4 groups (n= 20/group): 1) Control no FITC-d; 2) FR no FITC-d; 3) Control FITC-d 4.16 mg/kg; 4) FR FITC-d 4.16 mg/kg. Control groups had *ad libitum* access to feed meanwhile FR groups were feed restricted for 24 h before sampling. Serum was collected 2.5h post gavage and diluted 1:5 or 1:10 to determine if a higher dilution factor would eliminate some of the background fluorescence. Readings were performed with a gain 70.

Experiment 2

Comparing two sampling collection times and different gain readings of serum FITC-d in a 24 h feed restriction model

In this experiment, all chickens received FITC-d (4.16 mg/kg) and samples were collected at 1 or 2.5 h post FITC-d administration. Eighty chickens were randomly assigned to one of 4 groups (n= 20/group): 1) Control FITC-d collected 1 h post gavage; 2) FR FITC-d collected 1 h post-gavage; 3) Control FITC-d collected 2.5 h post-gavage; 4) FR FITC-d collected 2.5 h post-gavage. Control groups had *ad libitum* access to feed, meanwhile FR groups were feed restricted for 24 h before sampling. Serum was diluted at 1:5 and readings were done with gains 30, 35, 40 and 45.

Experiment 3

Comparing collection time of serum FITC-d diluted 1:5 and read at gain 40 in a 24 h feed restriction model

In this experiment, all chickens received FITC-d (8.32 mg/kg) and samples were collected at 1 or 2.5 h post FITC-d administration. Eighty chickens were randomly assigned to one of 4 groups (n= 20/group): 1) Control FITC-d collected 1 h post-gavage; 2) FR FITC-d collected 1 h post-gavage; 3) Control FITC-d collected 2.5 h post-gavage; 4) FR FITC-d collected 2.5 h post-gavage. Control groups had *ad libitum* access to feed, meanwhile FR groups were feed restricted for 24 h before sampling. Serum was diluted at 1:5 and readings were done using gain 40.

Experiment 4

Comparing the old method versus optimized method of serum FITC-d in a 24 h feed restriction model

The objective of this experiment was to compare our previous FITC-d method to the new optimized FITC-d method. Eighty chickens were randomly assigned to one of 4 groups (n= 20/group): 1) Control FITC-d (4.16 mg/kg) collected 2.5 h post-gavage.; 2) FR FITC-d (4.16 mg/kg) collected 2.5 h post-gavage; 3) Control FITC-d (8.32 mg/kg) collected 1 h post-gavage; 4) FR FITC-d (8.32 mg/Kg) collected 1 h post-gavage. In the old method serum was diluted 1:5, fluorescence measurements were quantified using an equation from a previously determined standard curve with known FITC-d concentrations using 0.9 % saline solution as a blank and measuring samples at gain 70. In the optimized method, serum from non-FITC-d chickens was obtained, to be used as a blank. Additionally, for each plate, a standard curve was adapted diluting

known concentrations of FITC-d in the 1:5 diluted blank sera as described above in the *in vitro* method. All serum samples were also diluted 1:5 for fluorescence reading at gain 40 (Table 5).

Statistical Analysis

All data were subjected to Analysis of Variance as a completely randomized design using the General Linear Models procedure of SAS (SAS Institute, 2002). In all trials, data are expressed as mean ± standard error. Significant differences among the means were determined by using Tukey's multiple-range test at $P < 0.05$.

Results

The results of the *in vitro* evaluation of different fluorescence gains using blank chicken sera, from chickens without FITC-d, versus 0.9% saline solution are summarized in Table 1. There was a significant difference between blank sera and 0.9% saline solution at each of the gains measured (40, 50, 60, 70, 80) (Table 1). This indicates that blank sera have a higher amount of fluorescence than 0.9% saline and is affected by the gain. Table 2 illustrates the results from Experiment 1 comparing two serum dilution methods on serum FITC-d (4.16 mg/kg) read at gain 70 in a 24 h feed restriction model. In this study, using the same sera, samples were diluted 1:5 and 1:10 to determine if a higher dilution factor would eliminate some of the background fluorescence. A significant reduction in the background fluorescence was observed in all samples diluted at 1:10 ($P < 0.05$). Interestingly, serum samples from FR chickens treated with FITC-d diluted at 1:5 or 1:10 showed significantly higher amounts of serum FITC-d concentration when compared with control chickens.

Results from Experiment 2 comparing two sampling collection times and different gain readings of serum FITC-d (4.16 mg/kg) in a 24 h feed restriction model are summarized in Table 3. Collecting the blood samples 1 h post FITC-d gavage not only showed significant increases in serum FITC-d concentration in chickens that received FR when compared with control chickens at all four gain readings, but the window of differences between feed restricted and control broilers were more evident when compared with serum collected at 2.5 h (Table 3).

Table 4 displays the results from Experiment 3, comparing collection time of serum FITC-d doubling the dose of FITC-d (8.32 mg/kg). Serum was diluted at 1:5 and read at gain 40 in a 24 h feed restriction model. These results confirmed and extended the results of Experiment 2. Sample collection time gives a stronger reading of serum FITC-d in FR chickens when is performed 1 h after FITC-d oral administration when compared with 2.5 h (Table 4).

The results from Experiment 4, comparing old method *versus* optimized method of serum FITC-d in a 24 h feed restriction model are summarized in Table 5. In the old method, chickens received 4.16 mg/kg FITC-d, serum samples were collected 2.5 h post gavage, samples were diluted 1:5 and fluorescence was measured using a previously determined standard curve, 0.9 % saline solution was used as a blank and measured at gain 70. No significant differences were observed between control and FR chickens. In contrast, in the optimized method, chickens receiving 8.32 mg/kg FITC-d, serum samples were collected 1 h post gavage, were diluted 1:5, non FITC-d serum was used as a blank, a standard curve was developed for each plate and a reading of gain 40, showed significant differences between control and FR chickens (Table 5).

Discussion

Stress is known to affect gastrointestinal tract (GIT) homeostasis by altering gut motility, permeability, as well as alterations in ion, fluid, and mucus secretion and absorption (Alverdy and Aoys, 1991; Collins and Bercik, 2009; Verbrugghe et al., 2011; Karavolos et al., 2013). Several investigators have reported that acute or chronic stress modify gut permeability associated with a temporary redistribution of tight junction (**TJ**) proteins (Maejima et al., 1984; Koh et al., 1996; Matter and Balda, 2007; Assimakopoulos et al., 2011). Some of these alterations are linked to Mast cells in the brain-gut axis which secrete several neurotransmitters and pro inflammatory cytokines, with profound effects on GIT physiology (Groschwitz and Hogan, 2009; Bailey et al., 2011; Lamprecht and Frauwallner, 2012). Another hormone that increases during acute or chronic stress is corticotropin releasing factor (CRF), which increases intestinal paracellular permeability via mast cell dependent release of TNF-α and proteases (Tache and Perdue, 2004; Teitelbaum et al., 2008; Overman et al., 2012). Moreover, excessive cortisol may lead to GIT disturbances, opportunistic infections, and impaired wound healing (Moeser et al., 2007; Smith et al., 2010; Galley and Bailey, 2014). Due to intensive selection, modern chickens are the most efficient meat-producing animals because of their fast growth, supported by a virtually unlimited voluntary feed intake. However, these features also cause many problems in breeder hens because of the negative correlation between muscle growth and reproduction effectiveness. Hence, commercial restricted feeding programs in broiler breeders have been implemented, with negative effects on welfare and health, as birds are continuously hungry (Decuypere et al., 2010). Previous research in poultry has shown that FR increases plasma levels of corticosterone causing disruption of gut barrier integrity, systemic and local inflammation (de Jong et al., 2003; Khajavi

et al., 2003; Hangalapura et al., 2005; Abu-Dieyeh, 2006)). Similarly, we have previously shown that intestinal barrier function can be adversely affected by stress, poorly digested diets (Tellez et al., 2014; Tellez et al., 2015), or feed restriction (Kuttappan et al., 2015b; Vicuña et al., 2015b), resulting in increased intestinal inflammation-associated permeability. In those studies, we have shown a correlation of liver bacterial translocation and serum concentrations of FITC-d as markers used to measure tight junction permeability. Fluorescein isothiocyanate dextran (FITC-d) is a 3 to 5kda marker used to measure tight junction permeability in chickens using enteric inflammation models. However, inconsistent results obtained from unpublished data suggested that current FITC-d methodology required further optimization. The results of the present study suggest that by increasing the dose of FITC-d (8.32 mg/kg *versus* 4.16 mg/kg); shortening the collection time of the blood (1 h *versus* 2.5); using a pool of non-FITC-d serum as a blank, compared to previously used 0.9% saline; generating a standard curve with every plate to set a limit of detection and modifying the software's optimal sensitivity value it is possible to obtain more consistent and reliable results.

Table 1. Evaluation of different fluorescence gain using blank chicken sera, from chickens without FITC-d, *versus* 0.9% saline solution. Non-FITC-d sera was diluted 1:5 in 0.9% saline onto black 96-well fluorescent plates and measured from gain 40 to 80.

	Gain 40	Gain 50	Gain 60	Gain 70	Gain 80
0.9% saline solution	1.0 ± 0.27 ^{b,z}	0.75 ± 0.31 ^{b,z}	12.6 ± 0.18 ^{b,y}	37.9 ± 0.74 ^{b,x}	93.1 ± 1.3 ^{b,w}
Blank sera	1.6 ± 0.11 ^{a,z}	1.1 ± 0.10 ^{a,z}	20.6 ± 0.38 ^{a,y}	59.8 ± 1.1 ^{a,x}	255.6 ± 6.6 ^{a,w}

$^{a, b}$ Superscripts within columns indicate significant difference at $P < 0.05$.

$^{w, x, y, z}$ Superscripts within rows indicate significant difference at $P < 0.05$.

Data is expressed as mean ± SE. n=20 birds/treatment.

Table 2. Comparing two serum dilution methods on serum FITC-d (4.16 mg/Kg) read at gain 70 in a 24 h feed restriction model to induce gut permeability in broiler chickens. Experiment 1

Experimental Group	Serum FITC-d (ng/mL) Diluted 1:5	Serum FITC-d (ng/mL) Diluted 1:10
Control No FITC-d	7.7 ± 2.7 [b, y]	1.0 ± 0.9 [b, z]
Feed restriction No FITC-d	11.4 ± 3.4 [b, y]	2.5 ± 1.6 [b, z]
Control FITC-d	9.1 ± 2.8 [b, y]	2.5 ± 1.2 [b, z]
Feed restriction FITC-d	23.1 ± 4.3 [a, y]	16.8 ± 3.1[a, z]

[a, b] Superscripts within columns indicate significant difference at $P < 0.05$.

[y, z] Superscripts within rows indicate significant difference at $P < 0.05$.

Data is expressed as mean ± SE. n=20 birds/treatment. In both comparisons, blanked serum was used to make a standard curve with every plate

Table 3. Comparing two sampling collection times and different gain readings of serum FITC-d (4.16 mg/Kg) in a 24 h feed restriction model to induce gut permeability in broiler chickens. Experiment 2

Experimental Group	Serum FITC-d (ng/mL) Gain 30	Serum FITC-d (ng/mL) Gain 35	Serum FITC-d (ng/mL) Gain 40	Serum FITC-d (ng/mL) Gain 45
Control FITC-d 1 h	0.00 ± .0.00 $^{c, z}$	61.0 ± 21.4 $^{c, y}$	49.6 ± 16.2 $^{c, y}$	56.3 ± 16.2 $^{c, y}$
Feed restriction FITC-d 1 h	207.5 ± 69.9 $^{b, y}$	284.4 ± 37.7 $^{b, x}$	185.6 ± 14.5 $^{a, z}$	177.1 ± 13.9 $^{a, z}$
Control FITC-d 2.5 h	390.8 ± 84.4 $^{a, x}$	257.0 ± 25.0 $^{b, y}$	118.9 ± 11.0 $^{b, z}$	95.4 ± 8.8 $^{b, z}$
Feed restriction FITC-d 2.5 h	468.1 ± 75.7 $^{a, x}$	336.5 ± 41.9 $^{a, y}$	191.3 ± 24.0 $^{a, z}$	153.3 ± 19.4 $^{a, z}$

$^{a, b}$ Superscripts within columns indicate significant difference at $P < 0.05$

$^{x, y, z}$ Subscripts within rows indicate significant difference at $P < 0.05$

Data is expressed as mean ± SE. n=20 birds/treatment. Serum was diluted 1:5. Blanked serum was used to make a standard curve with every plate.

Table 4. Comparing collection times of serum FITC-d (8.32 mg/Kg) diluted 1:5 and read at gain 40 in a 24 h feed restriction model to induce gut permeability in broiler chickens. Experiment 3

Experimental Group	Serum FITC-d (ng/mL)
Control 1hr	78.7 ± 9.4 [c]
Feed restriction 1 h	136. 5 ± 7.3 [a]
Control 2.5 h	67.1 ± 7.9 [c]
Feed restriction 2.5 h	112.4 ± 6.5 [b]

[a, b, c] Superscripts within columns indicate significant difference at $P < 0.05$

Data is expressed as mean ± SE. n=20 birds/treatment.

Table 5. Comparing old method *versus* optimized method of serum FITC-d in a 24 h feed restriction model to induce gut permeability in broiler chickens. Experiment 4

Experimental Group	Serum FITC-d (ng/mL)	Serum FITC-d (ng/mL)
	Old Method	**Optimized Method**
Control	306.0 ± 41.1 [a, y]	101.8 ± 36.0 [b, z]
Feed restriction	388.0 ± 28.0 [a, z]	397.3 ± 22.1 [a, z]

	Old Method	**Optimized Method**
FITC-d dose	4.16 mg/kg	8.32 mg/kg
Sample collection time	2.5 h	1 h
Blanked with	0.9% saline solution	Serum from non FITC-d chickens
Standard curve	Same	New with every plate
Serum dilution	1:5	1:5
Fluorescence reading	Gain 70	Gain 40

[a, b] Superscripts within columns indicate significant difference at $P < 0.05$.

[y, z] Superscripts within rows indicate significant difference at $P < 0.05$.

Data is expressed as mean ± SE. n=20 birds/treatment.

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Assessment of a Nutritional Rehabilitation Model in two modern broilers and their Jungle Fowl ancestor: A model for better understanding childhood undernutrition

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Abstract

This article is the first in a series of manuscripts to evaluate nutritional rehabilitation in chickens as a model to study interventions in children malnutrition and will include data on growth performance, bone mineralization and intestinal morphometric analysis. Inclusion of rye in poultry diets induces a nutritional deficit that leads to increased bacterial translocation, intestinal viscosity, and decreased bone mineralization. However, it is unclear the effect of diet on developmental stage or genetic strain. Therefore, the objective was to determine the effects of a rye diet during either the early or late phase of development on performance, bone mineralization and intestinal morphology across 3 diverse genetic backgrounds. Modern 2015 (Cobb 500) broiler chicken, 1995 Cobb broiler chicken, and the Giant Jungle Fowl were randomly allocated into four different dietary treatments. Dietary treatments were 1) a control corn-based diet throughout the trial (corn-corn); 2) an early phase malnutrition diet where chicks received a rye-based diet for 10 days, and then switched to the control diet (rye-corn); 3) a malnutrition rye-diet that was fed throughout the trial (rye-rye); and 4) a late phase malnutrition diet where chicks received the control diet for 10 days, and then switched to the rye diet for the last phase (corn-rye). At ten days of age, chicks were weighed, and diets were switched in groups 2 and 4. At day 20 of age, all chickens were weighed and euthanized to collect bone and intestinal samples. Body weight, weight gain and bone mineralization were different across diet, genetic line, age and all 2 and 3-way interactions ($P<0.05$). Overall, Jungle fowl were the most tolerant to a rye-based diet, and both the modern and 1995 broilers were significantly affected by the high rye-based diet. However, the 1995 broilers consuming the rye-based diet appeared to experience more permeant effects when compared to the modern broiler. The results of the present study suggest that chickens have a great potential as a nutritional rehabilitation model in human trials. The 1995 broilers line was an intermediate genetic

line between the fast-growing modern line and the non-selected Jungle Fowl line, suggesting it would be the most appropriate model to study for future studies.

Keywords: Nutritional rehabilitation, chicken lines, compensatory growth, bone mineralization, morphometric analysis

Introduction

Malnutrition is a growing concern as the global population continues to increase due to the increased global demand for food and the long-term effects of malnutrition. Malnutrition is due to the lack of, inadequate nutrition or inadequate absorption of nutrients. It has been identified as the underlying cause of death in one-third of children under 5 years of age. Of these deaths, 83% are attributed to mild-to-moderate malnutrition compared to severe malnutrition (Pelletier et al., 1995), suggesting that while malnutrition plays a major role in child mortality current strategies involving only the treatment of the severely malnourished may not be enough to reduce the negative impacts of malnutrition (Barendregt et al., 2008; Smith et al., 2013; Ahmed et al., 2014).

The most common manifestation of chronic malnutrition is stunted growth. It is estimated to affect 165 million children under the age of 5 years in low and middle-income countries (Schwarz et al., 2008). The critical period when stunting can develop is between pregnancy and the first 2 years of life (the first 1000 days) (Kuklina et al., 2006; Christian et al., 2013) and can be temporary or permanent. If the nutrient restriction is severe enough, permanent stunted growth may occur (Pelletier et al., 1995). While stunting is an outward consequence malnutrition, early life malnutrition may adversely affect brain anatomy, physiology, biochemistry, or lead to permanent brain damage (de Souza et al., 2011). Currently, strategies to prevent malnutrition focus on providing proper nutrition to overcome the condition, but research has rarely examined the consequences of these feeding strategies which may be due to limited availability of models.

Corn is the main energy source in poultry diets. However, it can be cost prohibitive to include in the diet at times. Unconventional grain sources can be used to reduce or replace corn usage during

times that are cost prohibitive. Unlike corn, rye contains a high amount of non-starch polysaccharides (NSP), which impairs nutrient digestion and absorption due to little or no intrinsic enzymes capable of hydrolysis of NSP in the small intestine. The increased NSP provide more nutrients in the ceca and large intestine which serve as a nutrient source for bacteria. The altered nutrient source can lead to dysbiosis within the gut. In poultry, rye-based diets increased both viscosity and *Clostridium perfringens* and *Clostridium difficile* proliferation when compared with corn-based diets (Latorre et al., 2015a). Additionally due to the anti-nutritive properties of rye, poultry consuming rye diets experience stunting and many similar pathologies associated with malnutrition in children including development of enteric enteropathy, alteration in gut microbiome profile, bacterial translocation, reduction in nutrient digestion and absorption as well as poor bone mineralization (Kolsteren et al., 1997; Soliman et al., 1986; Humphrey, 2009; Korpe and Petri, 2012; Ahmed et al., 2014; Petri et al., 2014; Tellez et al., 2014,2015; George et al., 2015). These similarities between chickens consuming rye diets and malnourished children may make poultry a potentially good model to understand short and long-term effects of malnourishment, however; it is unclear how selection practices in the broiler industry may alter these effects.

Undernutrition of children has profound effects on health and development; nevertheless the issue is not simply caused by a lack of food, but results from complex interactions of intra- and intergenerational factors (Wilson and Osbourn, 1960; Ahmed et al., 2014). Research on human nutrition has relied heavily on animal models for its insights (Delany, 2004; Baker, 2008). Avian models, specifically in the chicken, have been essential in contributing to the current understanding of several nutrient deficiencies, nutrient interactions, bioavailability, digestibility,

tolerances, and toxicities (Mozdziak and Petitte, 2004; Stern, 2004; Wolpert, 2004). Basic mechanisms of the enteric nervous system, the gut associated lymphoid tissue, and intestinal permeability is highly conserved across animal species. However, there are gastrointestinal physiological similarities between chickens and humans that make chickens a viable nutritional model when studying human nutrition: both species lipogenesis primarily takes place in the liver, iron is absorbed in the duodenum and neonatal humans and chickens can utilize sucrose as energy source (Pourquié, 2004; Raya and Izpisua Belmonte, 2004; Wittler and Kessel, 2004; Nafikov and Beitz, 2007). Lastly, in contrast with other animal models, chickens consuming diets high in non-starch polysaccharides (NSP) developed severe gut inflammation, accompanied with dysbacteriosis, decreased nutrient absorption, poor bone mineralization and increased liver bacterial translocation (Choct et al., 2010; Tellez et al., 2014). Some of these clinical signs are similar to what patients with environmental enteropathy (EE) experience. EE is an enigmatic disorder that often occurs in young children living in unsanitary conditions (Humphrey, 2009; George et al., 2015). Also, EE is characterized by reduced intestinal absorptive capacity, altered gut barrier function, intestinal inflammation, and dysbacteriosis (Korpe and Petri, 2012; Petri et al., 2014). Therefore patients with EE and chicks consuming diets high in NSP develop similar physio-pathology (Tellez et al., 2014,2015), making chickens a viable model when determining the effects of diet on childhood malnutrition.

Following nutrition deprivation, many organisms can undergo accelerated growth to return to a normal weight range, or also referred to compensatory growth (Wilson and Osbourn, 1960; Zubair and Leeson, 1996; Yair and Uni, 2011; Zhan et al., 2007). In some instances, body weight (BW) of animals under feed restriction will catch-up to control animals with *ad libitum* feed intake

(Wilson and Osbourn, 1960; Yair and Uni, 2011). In fact, high compensatory growth rates in feed restriction animals result in overcompensation due to excessive fat deposition and animals recover to normal weight without additional time (Zubair and Leeson, 1996; Zhan et al., 2007). Nevertheless, when the nutrient restriction is severe, the growth period must be extended to reach the normal weight, but if the nutrient restriction is severe enough, permanent stunted growth may occur (Pelletier et al., 1995). Some of the factors that affect compensatory growth include composition of the restricted diet, severity of undernutrition, duration of the period of undernutrition, age, genotype, and gender among others (Zulkifli et al., 2001,2009; Radder et al., 2007; Prabhakaran et al., 2008). Therefore, understanding the effects of compensatory growth following nutritional deficiencies could allow for strategies to be developed to mitigate the long-term effects of early childhood malnutrition.

Genetic selection has made modern broilers a unique model for understanding growth. It has allowed broiler chickens to double their starting BW in 3 days, and reach puberty in 4.5 months (Burt, 2004; Stern, 2005). Additionally, extensive work has been done to determine optimal nutrition and management for growth due to the increased pressures to improve and maintain high efficiency in agriculture production. In addition to modern poultry lines, the University of Arkansas have preserved minimally or unselected poultry lines. The Red Jungle Fowl is the closest living ancestor to the modern chicken and can be considered as a 'wild type' in poultry genomics (Wall and Anthony, 1995). Previous research determined that jungle fowl took 93 days longer to reach the same physiological BW than broiler breeders and had significantly lower average daily gain (Wall and Anthony, 1995). As well, the University of Arkansas maintains a random bred control line, which is the product of intercrossing 13 commercial broilers, parent lines from 1997

(Harford et al., 2014). This unique set-up allows for researcher to determine the effects of slight or severe malnutrition and nutritional recovery on performance and physiology. Therefore, we wanted to determine the effects of a malabsorptive diet during an early or a late growth phase on growth and bone and intestinal development across diverse genetic backgrounds. For this study, we utilized a rye diet which has been shown to induce nutritional deficiencies (Campbell et al., 1983; Bedford and Classen, 1993; Bedford and Schulze, 1998; Shirzadi et al., 2010, Tellez et al., 2014) when compared to a control corn diet across a modern commercial broiler, a commercial broiler of 1995 genetics, and an unselected Jungle Fowl line.

Materials and Methods

Animal source, diets and experimental design

All animal procedures were approved and in compliance with Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville (protocol #15006). The 3 lines of chickens were included in this study. For the modern broiler chickens, one hundred and sixty-one-day-old mixed broiler chicks, Cobb-Vantress, Silom Springs, AR, USA were used (n = 40 chickens/group). For the 1995 broiler chickens, one hundred and twelve one-day-old mixed broiler chicks, from the random bred line initiated from 1995 Cobb broiler chicken line (Harford et al., 2014) were used (n = 28 chickens/group). And for the Jungle fowl chickens, one hundred and sixty one-day-old mixed Giant Jungle Fowl (Gyles et al., 1967) were used (n = 40 chickens/group). On the day of hatch, chickens were neck-tagged, weighed and randomly allocated to one of four dietary treatment groups in floor pens containing new pine shavings in an environmentally controlled room. All diets were antibiotic-free and formulated to meet or exceed the current broiler nutritional requirements according to the National Research Council (1994; Table 1). When

administering dietary treatment, the experiment was split into two phases, the first phase was from day of hatch to d 10 and the second phase was from d 10 to d 20. Dietary treatments were 1) a control diet where chicks were maintained on a corn-based diet throughout the trial (corn-corn); 2) an early phase malnutrition diet where chicks were on a rye-based diet for 10 d, and then switched to the control diet (rye-corn); 3) a malnutrition rye-diet that was fed throughout the trial (rye-rye); and 4) a late phase malnutrition diet where chicks received the control diet for 10 d, and then switched the rye diet for the last phase (corn-rye) (Figure 1). Temperature was maintained according to normal management practices (34°C for the first 5 d then gradually reduced to 23°C). Individual BWs were recorded at day of hatch, d 10 and 20. Body weight gain was calculated by subtracting the initial BW from the final BW and was calculated from day of hatch to d 10 and from d 10 to d 20. Chickens were euthanized via carbon dioxide asphyxiation and samples were collected for bone and intestinal measurements on day 20.

Bone Parameters

Tissue was removed from the both the left and right tibias from each chicken (n = 10/group). Bone ash was measured on the left tibia according to published methods (Zhang and Coon, 1997). Briefly, bones were dried at 100°C for 24 h and weighed, then ashed in a muffle furnace (Isotemp muffle furnace, Fisher Scientific, Pittsburgh, PA) at 600°C for 24 h, cooled in a desiccator, and weighed. Bone mineral analysis was conducted for bone calcium and phosphorus content in the left tibia as well, using standard AOAC guidelines (AOAC International, 2000). For breaking strength, the right tibial diaphysis were cleaned of adherent tissues, the periosteum removed, and the biomechanical strength of each bone was measured using an Instron 4502 (Norwood, MA) with a 509 kg load cell using recommended protocols. Bones were held in identical positions. The mid-diaphyseal diameter of the bone at the site of impact was measured using a dial caliper. The

maximum load at failure was determined using a three-point flexural bend fixture with a total distance of 30 mm between the two lower supporting ends. The load, defined as force in kg per mm^2 of cross-sectional area (kg/mm^2). We will refer to this as bone strength. The rate of loading was kept constant at 20 mm/min collecting 10 data points per second using Instron's Series IX Software (Norwood, MA).

Histology and morphometric analysis of the intestine

Intestinal sections (0.5 cm) were collected from the middle of the descending duodenal loop, and at Meckel's diverticulum. These sections will be referred to as the duodenum and ileum, respectively. Tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (5-µm thick), and stained with hematoxylin and eosin (H&E), then examined by light microscopy. Photomicrographs of random chosen fields of each intestinal section were acquired using a microscope equipped with a Leica DFC450C camera and Leica V 3.8.0. software (Leica Application Suit). ImageJ 1.47v software was used for the morphometric analysis of villi height, crypt depth and muscularis width (Schneider et al., 2012). For each sample, ten measurements were taken. Measurements from the villus height (VH) and crypt depth (CD) were used to calculate the villus height to crypt depth (VH:CD) ratio.

Statistical analysis

Data were analyzed using a linear mixed model procedure in SAS (PROC MIXED; SAS Institute Inc., 2002) where the factors of dietary treatment, genetic line, and the interaction between the dietary treatment and genetic line were fit as fixed effects for all variables. Since birds were weighed multiple times, a repeated effect for day was fit when analyzing body weight and body weight gain data, and age of the bird along with the 2- and 3- way interactions were included at fixed effects in the statistical model. Significance was set at a $P < 0.05$. When factors were

determined to be significant, pairwise comparisons were performed in SAS using the LSMEANS statement and corrected for multiple tests using Tukey post-hoc adjustment.

Results

Body weight

The results of the evaluation of a nutritional rehabilitation model on BW in three genetic chicken lines fed rye or corn at varying time points are summarized in Table 2. As to be expected, BW increased as birds aged ($P<0.0001$) regardless of dietary treatment and genetic line. There was a significant difference in BW between all treatment groups, where the corn-corn group ($P<0.0001$) had the highest BW, and the rye-rye group ($P<0.0001$) had the lowest body weight. Modern broilers were the heaviest, followed by the 1995 broilers and then the jungle fowl ($P<0.0001$).

Regardless of dietary treatment there was a significant increase in BW from d 1 to d 10 and d 10 to d 20 in the jungle fowl, 1995 broilers and the modern broiler. Overall, there was a significant dietary treatment by genetic lines interaction ($P<0.0001$), where modern broilers in the corn-corn group were heaviest ($P<0.0001$) and jungle fowl in the rye-rye group were the lightest ($P<0.0001$). Across genetic line at both d 10 and d 20, the modern broilers were heavier than the 1995 broiler or the Jungle Fowl for all treatment groups ($P<0.0001$). Also, 1995 boilers weighed more than the Jungle fowl for all treatments groups ($P<0.0001$). Genetic line had a significant effect on BW at 9 d of age where regardless of diet, modern broilers weighed more than the 1995 broilers and jungle fowl ($P<0.0001$) and 1995 broilers weighed more than Jungle fowl ($P<0.0001$).

At d 10, modern broiler in the rye-rye treatment group had significantly lower BW than the corn-corn ($P=0.0016$) and corn-rye ($P=0.0106$). There was no difference in BW between treatment groups consuming the same diet (corn-corn vs corn-rye and rye-rye vs rye-corn; $P=1.00$).

Surprisingly, there was also no difference in BW between rye-corn treatment group and the corn-corn (P=0.2104) and the corn-rye (P=0.2979) treatment groups. However, during the first phase of the experiment chicks in the corn-corn and corn-rye were both consuming a corn-based diet, just as chicks on the rye-rye and rye-corn groups were both consuming a rye based diet. Therefore, when BW of chicks consuming the same diets were combined (corn-corn with corn-rye and the rye-rye with rye-corn), corn fed chicks weighed significantly more than rye fed chicks (P<0.0001; Table 2). At d 10, 1995 broilers in the rye-corn treatment group had significantly lower BW than the corn-corn (P=0.0002) and corn-rye (P=0.0008). There was no difference in BW between the corn-corn and corn-rye treatment groups (P=1.000) nor was there a difference in BW between the rye-rye and rye-corn treatment groups (P=0.8282). Similar to what was observed in the modern broilers, there was also no difference in BW between rye-rye treatment group and the corn-corn (P=0.412) and the corn-rye (P=0.6993) treatment groups. However, when BW of chicks consuming the same diets were combined (corn-corn with corn-rye and the rye-rye with rye-corn), corn fed chicks weighed significantly more than rye fed chicks (P<0.0001; Table 2). There was no difference in BW between treatments in Jungle Fowl at d 10.

At d 20, Modern broilers in the corn-corn and rye-corn groups did not differ in BW (P=0.8935) but weighed significantly more than those fed rye in the second phase of the experiments (P<0.0001). Also, modern broilers in the corn-rye group weigh significantly more than the rye-rye fed chicks (P<0.0001). Body weight of 1995 broilers at 20 days of age were significantly different between all treatment groups, where the corn-corn group weighed significantly more than those fed rye at any phase of the experiment (P<0.0001). The rye-corn group weighed significantly more than the rye-corn and the rye-rye group (P<0.0001). 1995 broiler in the rye-rye group had

significantly lower BW than the corn-rye group (P=0.0007). At d 20, jungle fowl in the corn-corn group weigh significantly more than the rye rye-group (P=0.0002) however there were no significant differences in BW between the other treatments. Within each dietary treatment there was a significant difference in BW, where modern broilers weighed more than both 1995 broilers and Jungle Fowl (P<0.0001), and 1995 broiler weighed more than Jungle Fowl (P<0.0001).

Body weight gain

Table 3 shows the evaluation of a nutritional rehabilitation model on average body weight gain (BWG) in three genetic chicken lines fed rye or corn at varying time points. As expected, BWG increased over time where BWG from d 10-20 was significantly higher than BWG from d 1-10 (P<0.0001). Dietary treatment had a significant effect on BWG (P<0.0001), regardless of day or genetic line, where chicks in the corn-corn group had significantly higher BWG than those fed rye at any phase of the experiment (P<0.0001). The rye-corn group had the second highest BWG which was significantly higher than those chicks fed rye in the second phase of the experiment (P<0.0001). Also, the corn-rye group had a significantly higher BWG than the rye-rye group (P<0.0001). Genetic line had a significant effect on BWG, where modern broilers had significantly higher BWG than the 1995 broiler (P<0.0001) and Jungle Fowl (P<0.0001). As well, 1995 broiler had significantly higher BWG than the Jungle Fowl (P<0.0001).

Interaction between the age of the chicks, dietary treatment and genetic line had a significant effect on the BWG (P<0.0001). At d 0-10, modern broilers in the rye-corn treatment group had significantly lower BWG than the corn-corn (P=0.0011) and corn-rye (P=0.0033). BWG from d 1-10 in modern broilers was significantly lower in the rye-rye treatment group when compared to the corn-corn (P=0.0233) and corn-rye (P=0.0205). There was no difference in BWG between

treatment groups consuming the same diet, (corn-corn vs corn-rye and rye-rye vs rye-corn; P=1.00) which was expected. There was also no difference in BWG between rye-corn treatment group and the corn-corn (P=0.4095) and the corn-rye (P=0.3831) treatment groups. However, when BWG of chicks consuming the same diets were combined for D1-10 (corn-corn with corn-rye and rye-rye with rye-corn), corn fed chicks had a significantly higher BWG than rye fed chicks (P<0.0001; Table 3). At d 0-10, 1995 broilers in the rye-corn treatment group had significantly lower BWG than the corn-corn (P=0.0011) and corn-rye (P=0.0033). There was no difference in BWG between the corn-corn and corn-rye treatment groups (P=1.000) nor was there any difference in BWG between the rye-rye and rye-corn (P=0.844). However, there was also no difference in BWG between rye-rye treatment group and the corn-corn (P=0.6934) and the corn-rye (P=0.8645) treatment groups. When BWG of chicks consuming the same diets were combined (corn-corn with corn-rye and the rye-rye with rye-corn), corn fed chicks weighed significantly more than rye fed chicks (P<0.0001; Table 3). BWG from d 1-10 was not statistically different between dietary treatment groups in Jungle Fowl. Between genetic lines, modern broilers fed corn or rye had significantly higher BWG from d 1-10 than the 1995 broiler (<0.0001) and Jungle fowl (P<0.0001). In addition, 1995 broilers fed corn in the first phase of the experiment had significantly higher BWG from d 1-10 than Jungle fowl (P<0.0001).

From d 10-20 in modern broilers there was no significant difference in BWG between the corn-corn and rye-corn treatment groups (P=0.9999) nor was there a statistical difference between the rye-rye and corn-rye groups (P=0.5103). Modern broilers in the corn-corn and rye-corn groups had significantly higher BWG than the rye-rye and corn-rye groups (P<0.0001). BWG from d 10-20 in the 1995 broiler line was statistically different between all treatment groups, where the corn-

corn group had the highest BWG (P<0.0001), followed by the rye-corn group (P<0.0001), then the corn-rye group (P<0.0001) and lastly the lowest BWG occurred in the rye-rye group (P<0.0001). From d 10-20 Jungle Fowl in the corn-corn group had significantly higher BWG than the rye-rye treatment group (P=0.0059) however there were no difference in BWG between any of the other treatment groups. There was significant differences in BWG between genetic lines in the corn-corn and rye-corn treatment groups from d 10-20, where the modern broilers had the highest BWG (P<0.0001), followed by the 1995 broiler (P<0.0001) and Jungle Fowl had the lowest BWG (P<0.0001). A similar trend was observed in the corn-rye groups, where modern broilers in the corn-rye group had the highest BWG (P<0.0001), followed by the 1995 broiler (P<0.0001), with the Jungle fowl having the lowest BWG (<0.0001). In the rye-rye group, modern broilers had significantly higher BWG than both the 1995 broiler and the Jungle Fowl (P<0.0001). 1995 broiler in the rye-rye treatment group also had significantly higher BWG than the Jungle Fowl (P=0.0039).

Bone parameters

Tibia strength

Table 4 summarizes the bone parameters of three different genetic lines of chickens lines fed rye or corn at varying time points at 20 days of age. Tibia strength between treatments, genetic lines and interaction between the two variables were statistically different (P<0.001). Modern chickens fed corn in the second phase of the experiment had significantly higher tibia strength when compared with rye fed chickens (P<0.0001). In the 1995 chicks, the corn-corn group had significantly higher tibia strength than those chicks fed rye in any phase of the experiment (P<0.0001). Chicks in the rye-corn group had significantly stronger tibias than the rye-rye (P<0.0001) and the corn-rye (P=0.0065) groups, however there were no difference in tibia strength

between the rye-rye and corn-rye groups (P=0.9511). There were no significant differences in tibia strength between treatments in the jungle fowl. When comparing genetic lines within each treatment group, the modern broilers and 1995 broilers had significantly stronger tibias than the jungle fowl in the corn-corn group (P<0.0001). Modern broilers had significantly stronger tibias than the 1995 broilers in the rye-rye (P=0.0465) and corn-rye group (P=0.0153). Lastly, in the rye-corn group, modern broilers had significantly stronger tibias than the 1995 broilers and jungle fowl (P<0.0001).

Tibia ash

At d 20, treatment, genetic line and interaction between the two variables had a significant effect on tibia ash (P<0.0001; Table 4). Modern and 1995 broilers fed corn in the second phase of the experiment had significantly higher tibia ash content than rye-fed chicks (P<0.0001). In the Jungle Fowl, there was no significant difference in tibia ash between the treatments. Between genetic lines, modern broilers had significantly higher tibia ash than the 1995 broilers in the rye-rye (P<0.0001) and corn-rye (P<0.0001) groups. Similar results were observed in the Jungle Fowl where the rye-rye (P=0.0083) and corn-rye (P=0.0016) groups had significantly higher tibia ash than the 1995 broiler. Modern broiler in the rye-corn group had significantly higher tibia ash than the 1995 broiler (P=0.0002) and the Jungle Fowl (P<0.0001). In the corn-corn group modern broilers had significantly higher ash content than the 1995 broilers (P=0.0167) and the Jungle Fowl (P<0.0001). As well, 1995 broiler had significantly higher tibia ash content than Jungle Fowl (P=0.034).

Calcium and phosphorus content

Modern broilers in the corn-corn group had significantly higher tibia calcium content than those chicks in the rye-rye ($P=0.0152$) and corn-rye ($P<0.0001$). Similar results were observed in rye-corn groups, where the modern broilers in the rye-corn group had significantly higher tibia calcium content than the rye-rye ($P=0.0004$) and the corn-rye ($P<0.0001$) groups. Modern broilers in the rye-corn group had higher phosphorus content than the corn-rye group ($P=0.0036$), however no differences were observed between the other treatment groups. In the 1995 broilers, the corn-corn group had significantly higher tibia calcium concentrations than the corn-rye group ($P=0.003$). The corn-rye group had significantly lower tibia phosphorus content than the corn-corn ($P=0.0064$) and rye-rye ($P=0.0493$) groups. There was no difference in calcium and phosphorus content between the other treatment groups in the 1995 broilers. There was no difference in calcium or phosphorus levels between treatments in the Jungle Fowl. Calcium content in the corn-corn group was significantly higher in the modern broiler compared to the 1995 broiler ($P=0.0045$) and the Jungle Fowl ($P=0.0499$). For the rye-corn group, modern broilers had significantly higher tibia calcium content than the Jungle Fowl ($P=0.004$) and the 1995 broiler line ($P=<0.0001$). In the corn-rye group, Jungle Fowl had significantly higher calcium content than the 1995 line ($P=0.0024$). Tibia phosphorus content in the corn-corn group was significantly higher in the modern broiler than the Jungle Fowl ($P=0.016$). In the rye-corn group the modern broiler had significantly higher phosphorus content in the tibia than the 1995 broiler ($P=0.0166$) or the Jungle Fowl ($P=0.0115$). In the corn-rye group, modern broilers had significantly higher tibia phosphorus content than the 1995 broiler ($P=0.0287$). There was no difference in calcium or phosphorus content between genetic lines for those birds maintained on the rye treatment throughout the experiment (Table 4).

Morphometric analysis

Table 5 shows the results of the evaluation of a nutritional rehabilitation model on morphometric analysis of the duodenum in three genetic chicken lines fed rye or corn at varying time points at day 20 of age.

Duodenum

Duodenal villus height was significantly affected by treatment ($P=0.0003$) and genetic line ($P<0.0001$); however, there was no significant interaction between the two variables ($P=0.059$). Regardless of genetic line, the corn-corn group had significantly shorter villi than the rye-rye ($P=0.0032$) and the corn-rye groups ($P=0.0004$). Overall, Jungle Fowl had significantly shorter villi than the modern broiler ($P<0.0001$) and the 1995 broiler ($P<0.0001$), however villus height was similar between the modern and 1995 broiler ($P=0.9009$). Duodenal crypt depth was altered by treatment, genetic line and the interaction between the two variables had a significant effect on duodenal crypt depth ($P<0.0014$). Modern broilers in the corn-corn and rye-corn treatment group had significantly shorter crypt depth than those fed rye-rye ($P<0.05$) or corn-rye ($P<0.05$). 1995 chickens in the corn-corn group had significantly shorter crypt depth than those chicks in the rye-rye ($P<0.0001$) and the corn-rye ($P=0.0001$). The 1995 chicks in the rye-corn group had significantly lower crypt depth than the rye-rye ($P=0.0006$) fed chicks however there was no differences in crypt depth between the corn-rye and rye-corn groups ($P=0.1236$). In the Jungle Fowl, there was no significant difference between treatments for duodenal villus height and crypt depth. There were no significant differences in crypt depth between genetic lines in chicks fed a corn-based diet in the second phase of the experiment. However, jungle fowl in the rye-rye and corn-rye group had significantly shorter crypt depth than the modern broiler ($P<0.005$) and 1995

broilers (P<0.005). In the duodenum, there were no differences between treatments, genetic lines or interaction between the treatment and genetic lines in the VH:CD (Table 5). Genetic line had a significant effect on muscularis thickness (P=0.0391) where modern broiler had a significantly thicker muscularis than the Jungle fowl (P=0.0480). Treatment (P=0.203) and interaction between the treatments and genetic lines were similar for muscularis thickness (P=0.0609).

Ileum

The results of the evaluation of a nutritional rehabilitation model on morphometric analysis of the ileum in three genetic chicken lines are summarized in Table 6. Unlike the duodenum, the ileum was relative unaltered by line, dietary treatment or the interaction of line by dietary treatment. A significant interaction was observed between the treatments and genetic line for ileal villus height (P=0.048), however, after multiple testing correction (Tukey multiple comparison test), no statistical differences were observed between treatments or genetic lines for ileal villus height. Jungle fowl villi were shorter than the modern broiler (P=0.0024) and the 1995 broiler (P=0.0301). Genetic line had a significant effect on villus height, crypt depth and muscularis thickness (P<0.01) where modern broilers had significantly taller villi, deeper crypt depths, and thicker muscularis compared to the 1995 broiler (P< 0.05) and Jungle Fowl (P<0.05). The 1995 broilers was in intermediate between Jungle Fowl and the modern broiler for all 3 traits (P=0.0003).

Dietary treatment had a significant effect on VH:CD ration, where the corn-corn group had a significant higher VH:CD ratio than the rye-rye (P=0.0002) and corn-rye (P=0.0003) groups. Similar results were observed in the rye-corn group, where the rye-rye (P=0.0292) and the corn-

rye (P=0.0467) groups had a significantly lower VH:CD ration. All other effects were not altered, and P-values can be found in table 6.

Discussion

Body weight /compensatory gain

Chickens may be the ideal animal for preclinical studies of growth because of their rapid growth rates and the extensive amount of literature on poultry nutrition (Burt, 2004; Mozdziak and Petitte, 2004; Tickle, 2004; Wolpert, 2004; Stern, 2005). It is evident in the present study that compensatory gain occurred in the modern broilers as the rye-corn treatment group had the same BW and BWG as those maintained a corn-based diet throughout the experiment. Interestingly, in the 1995 broilers, the rye-corn treatment group exhibited compensatory growth as they had a higher BW and BWG than those chicks in the corn-rye group. However, 1995 broilers in the corn-corn treatment group weighed significantly more than the rye-corn group suggesting there was not a full recovery within the observed timeframe. The slower compensatory growth rate observed in the 1995 broilers was similar to that of stunted children in developing countries, where the effects of stunting are often permanent after 3 years of age (Shrimpton et al., 2001). Therefore the 1995 broilers may be the most appropriate model organism when determining clinical interventions. The difference in BW and BWG between the modern broiler and the 1995 broiler in the rye-corn group also suggests that genetic selection within the last 20 years has allowed modern broilers to exhibit compensatory gain after a period of undernutrition. Similar results were observed by Zubair and Leeson (1996) who reported that re-feeding broilers after quantitative or qualitative feed restriction allowed for some BW to be recovered but BW was not fully compensated (Zubair and Leeson, 1996). Interestingly, this article was published in 1996, so potentially these birds may have had a similar genetic profile to the 1995 line used in this current trial (Zubair and Leeson, 1996). In the

Jungle Fowl, there were no significant differences in BW between the treatments at day, 10 or 20. When Jungle Fowl were placed on a choice fed diet, they choose nutrients to support optimum growth (Zulkifli et al., 2001), suggesting that Jungle Fowl are not adapted to a particular diet and they eat to their metabolic requirements. Since both the rye-based diet and corn-based diet were isonitrogenous, perhaps both the diets contained the protein levels to meet maintenance and growth requirements of Jungle Fowl. Furthermore, similar to Leghorn chickens, Jungle Fowl eat until their energy and protein requirements are met (Zulkifli et al., 2001). In contrast, broilers eat until the gut capacity is full (Nir et al., 1993). However, Jungle Fowl in the corn-corn group had significantly higher BWG than the rye-rye group, suggesting that the anti-nutritional factors of a rye-based diet also affected these chickens. As far as we are aware, this is the first study performed looking at the effect on high NSP diets on compensatory gain in chickens. Previous research used quantitative or qualitative feed restriction to study compensatory gain (Yu and Robinson, 1992; Zubair and Leeson, 1994). Rosebrough and McMurtry (1993) placed broilers on short term energy restriction from 6-12 days of age and the allowed *ad libitum* access feed, which led to compensatory gain. Zhan et al. (2007) reported that the average daily gain was significantly lower when chickens were feed restricted for 4 hr from 1-21 days of age. However, once the chicks had *ab libitum* access to feed there was no significant difference in BW or average daily gain, attributing the compensatory gain to the reduction in basal metabolic rate by reducing the amount of thyroid hormone (Zhan et al., 2007). In addition, BW and BWG were highest in modern broilers, followed by 1995 chickens, and Jungle Fowl had the lowest body weight. These results are in agreement with Wall and Anthony (1995) reporting that it takes 93 days for Jungle Fowl chickens to reach the same BW of broiler breeders. Genetic selection for various production traits has reduced genetic diversity in broilers and laying hens (Siegel et al., 1992). This may be a

contributing factor as to why the broilers in the current study were more affected by the anti-nutritional factors in a rye-based diet when compared to the Jungle Fowl.

From the present trial, it is evident that chicks maintained on rye-based diet throughout had significantly lower BW and BWG in modern and 1995 broilers especially when compared to Jungle Fowl. This is because whole rye contains of a high amount of NSP, like β-glucan and arabinoxylans. These soluble NSP absorb water and increasing digesta viscosity which impairs nutrient digestion and absorption. The lack of nutrient digestion and absorption in the small intestine allows more nutrients to enter the lower intestine, providing bacteria a nutrient source and leading to dysbiosis within the gut. Hence, rye diets evoke mucosal damage in chickens that alter digesta viscosity, increase leakage throughout the intestinal tract and affect the microbiota composition as well as bone mineralization (Tellez et al., 2014,2015). Studies published by our laboratories have shown that rye-based diets significantly increased both viscosity and *Clostridium perfringens* and *Clostridium difficile* proliferation when compared with corn-based diets (Latorre et al., 2015a). Since poultry have little or no intrinsic enzymes capable of hydrolyzing these NSP, exogenous carbohydrases are used as additives to reduce the negative impact of these anti-nutritive factors. Many of the clinical and pathological effects reported in poultry consuming diets high in NSP are also observed in stunted children living in low-income countries (Korpe and Petri, 2012; Petri et al., 2014). These include, stunting, development of enteropathy, alteration in gut microbiome profile, bacterial translocation, reduction in nutrient digestion and absorption as well as poor bone mineralization (Soliman et al., 1986; Kolsteren et al., 1997; Humphrey, 2009; Korpe and Petri, 2012; Ahmed et al., 2014; Petri et al., 2014; George et al., 2015). This suggests that a

rye-based diet is a viable approach to induce undernutrition in chickens to study compensatory growth and clinical interventions in malnourished children.

Bone health

The increase intestinal viscosity caused by high NSP diets impairs digestion and absorption of nutrients (Bedford et al., 1991). Furthermore, the high concentration of phytate in rye has been linked to contribute to the poor bone mineralization (Martos et al., 2010; Choct, 2006). Nevertheless, supplementation of enzymes or DFM that produce exogenous enzymes ameliorate these negatives effects (Bedford and Classen, 1993; Latorre et al., 2014; Latorre et al., 2015b). Modern broilers fed corn in the second phase of the experiment had stronger tibias, higher amounts of tibia ash and higher levels of calcium than broilers fed rye. This indicates that chicks in the rye-corn group were able to recover bone parameters within the observed time frame. The 1995 broilers fed only corn had significantly stronger tibias than any of the other treatment groups including the rye-corn group. However, 1995 broilers in the rye-corn group had stronger tibias than those birds fed rye in the second phase of the experiment indicating some recovery in bone strength has occurred. Tibia bone ash improved when a low phosphorus diets were supplemented with phytase, suggesting that the enzyme increased mineral availability (Pieniazek et al., 2016). Therefore, it likely that switching from a rye to a corn-based diet increased mineral availability allowing for improved bone quality in modern and 1995 broilers. Modern broilers fed corn had significantly more tibia ash and calcium than the 1995 broiler and Jungle Fowl. This further supports the idea the modern broilers can recover from insult faster and may even be more resistant to the anti-nutritional factors in a high NSP diet. Similarly, Jungle Fowl chickens, fed in the corn-corn group had significantly stronger tibias than those fed rye at any phase of the experiment. This finding did

not correlate with the performance data where there were no differences in BW or BWG between treatments. This suggests that Jungle Fowl chickens consuming a rye-based diet at any phase of the experiment may be meeting energy requirements for growth, but mineral deficiencies may be affecting bone strength. However, there was no difference between treatments for percent tibia ash, or calcium and phosphorus levels.

Histology

Selection for growth has altered the morphology of the gastrointestinal tract to accommodate nutrient absorption for rapid growth (Smith et al., 1990). In the current experiment, there were no significant interaction between dietary treatments and genetics for villus height, VH:CD and muscularis in the duodenum. These results are similar to previous studies showing that wheat diets increased duodenal digesta viscosity but had no effect on morphology of the gastro intestinal tract (Wu et al., 2004; Pirgozliev et al., 2010). In the duodenum, the chicks fed corn in the second phase of the experiment had statistically lower villi than rye fed chicks. Previous research has found that chickens consuming diets high in fiber increase epithelial turnover with the consequent increase in villus height, as a measure to try to compensate for the poor digestibility (Jin et al., 1994; Yu and Chiou, 1996; Zulkifli et al., 2009). Other studies have demonstrated that removing various sections of the small intestine had little effect on BW, nitrogen retention, dry matter digestibility, due to hypertrophy of intestinal villi and epithelial cells in the remaining small intestine (Yamauchi et al., 2010). Therefore, chickens fed rye in the second phase of the experiment may be trying to acquire more nutrients by increasing villus height in an attempt to increase nutrient intake.

Crypt depth is an indicator of cell turnover rate, and shorter villi and deeper crypts are often indicators of toxin present in the lumen of the gastrointestinal tract (Yason et al., 1987; Xu et al.,

2003). The more energy required for cellular replication, the less is available for growth (Xu et al., 2003). Modern broilers fed corn in the second phase of the experiment had significantly shallower crypt depth in the duodenum than those chicks fed rye in the second phase of the experiment. This data follows a similar trend to that of the BW and bone data, where chicks in the rye-corn group can recover and decrease crypt depth after consumption of a rye-based diet. Previous work conducted in our laboratory suggest that the dysbacteriosis and high number of coliforms associated with rye diets in poultry is a source of toxins that not only induce intestinal inflammation and gut permeability, but also deeper crypts (Tellez et al., 2014, 2015). In the duodenum, rye-fed broilers had deeper crypt but there was no significant difference in villus height between treatments. Therefore, the intestinal epithelial cells have a high turnover rate but the same villus height. Similar results have been reported where diets containing soluble NSP resulted in deeper crypts by shorter villi in the ileum (Rahmatnejad and Saki, 2016). The short villi may be due to increased apoptosis when consuming a diet high in soluble NSP (Teirlynck et al., 2009). Overall muscularis thickness in the duodenum was significantly higher in the modern broilers when compared to Jungle Fowl however there was no difference in muscularis thickness between dietary treatments. This may indicate that modern broilers have more intestinal inflammation of the muscularis. Recent studies in broiler chickens fed with a rye/wheat-based diet had T-cell aggregates in the mucosa as an indicator of excessive immune stimulation as well as a thinner tunica muscularis when compared to those fed a maize-based diet, suggesting that excessive stimulation of the immune system led to thin muscularis, which may explain why there were no differences in muscularis thickness treatment groups (Teirlynck et al., 2009).

There was no significant interaction between dietary treatments and genetics for crypt depth, VH:CD and muscularis in the duodenum. The VH:CD ratio was significantly higher in corn fed chicks when compared to rye fed chicks in the ileum. Comparable results were observed by Mathlouthi et al. (2002) for the VH:CD ratio in corn-fed chickens had significantly higher ratio than the rye-fed birds, but the addition of enzymes improved villus height to that of the corn-fed birds (Mathlouthi et al., 2002). The authors hypothesized that supplementation of enzymes reduced digest viscosity which led to longer villus, hence increasing broiler performance (Mathlouthi et al., 2002). In our study, we also observed that switching from a rye to a corn-based diet improved VH:CD ratio. However, in the current experiment the improvement was not due to increased villus height but a decrease in crypt depth. Although digesta viscosity was not recorded in the current experiment, previous research published by our laboratory using the same diet formulation reported high digesta viscosity in a rye-based diet (Tellez et al., 2014,2015).

Regardless of dietary treatment, modern broilers fed rye in any phase of the experiment had significantly deeper crypt depth, longer villi and thicker muscularis than the Jungle Fowl in both the duodenum and ileum. Zulkifli et al. (2009) attributed the difference in intestinal size and surface area to the different growth rates (Zulkifli et al., 2009). Although we did not measure intestinal length, Wall and Anthony (1995) found that broiler breeds had heavier and longer digestive tracts than the Jungle Fowl, suggesting that selection for feed efficiency affects total size of the gastrointestinal tract. It has also been reported that is a positive correlation for growth rate and digestion and absorption of nutrient (Chambers et al., 1981).

Conclusion

It is evident from the present study that the consumption of a rye-based diet had malabsorptive effects on broilers, especially the in the 1995 broiler in the rye-corn group as they were unable to fully recover BW and tibia strength. The results of the present study suggest that chickens are a viable model to study nutritional rehabilitation in human trials. Specifically, the 1995 chicken line exhibited a compensatory gain patterns to that observed in malnourished children. Our next objective is to design a nutritional trial evaluating our DFM spore base probiotic (that produce exogenous cellulase, xylanase, amylase, β-galactase, phytase, proteases, and lipases) alone or in combination with selective lactic acid-based probiotic, using this model in 1995 chickens, before we expand our trials in children to test the lessons learned from our pre-clinical model. This process would include identifying a site in sub-Saharan Africa where these interventions could be tested in children with EE, ensuring that all of the human subject's protections are in place, and developing the best strategies for measuring clinical outcomes.

Table 1. Composition and nutrient content of the experimental diets (%)

Item Ingredients (%)	Rye-based diet	Corn-based diet
Corn	-	57.32
Rye	58.27	-
Soybean meal	31.16	34.66
Poultry fat	6.30	3.45
Dicalcium phosphate	1.80	1.86
Calcium carbonate	1.10	0.99
Salt	0.38	0.38
DL-Methionine	0.35	0.33
Vitamin premix[1]	0.10	0.20
L-Lysine HCl	0.22	0.31
Choline chloride 60%	0.10	0.20
Mineral premix[2]	0.12	0.12
Threonine	0.08	0.16
Antioxidant[3]	0.02	0.02
Calculated analysis		
Metabolizable energy (kcal/kg)	2850	3035
Crude protein, %	22.38	22.16
Lysine, %	1.32	1.35
Methionine, %	0.64	0.64
Methionine + Cystine, %	0.98	0.99
Threonine, %	0.86	0.91
Tryptophan, %	0.30	0.28
Total calcium, %	0.90	0.9
Available Phosphorus (%)	0.45	0.45
Sodium (%)	0.16	0.16

[1]Vitamin premix supplied the following per kg: vitamin A, 20,000 IU; vitamin D3, 6,000 IU; vitamin E, 75 IU; vitamin K3, 6.0 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 µg; and ascorbic acid, 200 mg (Nutra Blend LLC, Neosho, MO 64850).
[2]Mineral premix supplied the following per kg: manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg (Nutra Blend LLC, Neosho, MO 64850).
[3]Ethoxyquin.

Table 2. Evaluation of a nutritional rehabilitation model on body weight in three genetic chicken lines fed rye or corn at varying time points.

Day	Treatment	Genetic Line			Variable	P-Value
		Modern Broiler	1995 Line	Jungle Fowl		
1	Corn	40.11 ± 0.33 [a, z, 3]	40.75 ± 0.39 [a, z, 3]	34.26 ± 0.33 [a, z, 3]	trt	<0.0001
	Rye	39.85 ± 0.33 [a, z, 3]	40.37 ± 0.40 [a, z, 3]	33.65 ± 0.33 [a, z, 3]	line	<0.0001
10	Corn	175.91 ± 1.73 [a ,x, 2]	123.71 ± 2.07 [a, y, 2]	74.45 ± 1.73 [a, z, 2]	day	<0.0001
	Rye	151.74 ± 1.76 [b, x, 2]	95.3 ± 2.11 [b, y, 2]	69.08 ± 1.77 [a, z, 2]	trt*line	<0.0001
20	Corn-Corn	715.5 ± 5.84 [a, x, 1]	528.07 ± 6.75 [a, y, 1]	190.05 ± 5.84 [a, z, 1]	trt*day	<0.0001
	Rye-Corn	695.85 ± 5.84 [a, x, 1]	340 ± 6.75 [b, y, 1]	165.4 ± 5.84 [ab, z, 1]	line*day	<0.0001
	Rye-Rye	393.59 ± 6.34 [c, x, 1]	231.2 ± 6.75 [d, y, 1]	143.45 ± 5.84 [b, z ,1]	trt*line*day	<0.0001
	Corn-Rye	453.8 ± 5.84 [b, x, 1]	280.88 ± 6.53 [c, y,1]	173.4 ± 5.84 [ab, z, 1]		

Data is expressed as the LSmean ±SE. [a-c] Indicates significant differences between the treatments within the column at each time point. [x-z] Indicates significant difference between the genetic lines within the rows at each time point. [1-3]Indicates significant difference between the collection days within the columns at each time point.

Table 3. Evaluation of a nutritional rehabilitation model on average body weight gain in three genetic chicken lines fed rye or corn at varying time points.

Day	Treatment	Genetic Line			Variable	P-Value
		Modern Broiler	1995 Line	Jungle Fowl		
1-10	Corn	135.80 ± 1.78 [a, x, 2]	81.46 ± 2.12 [a, y, 2]	40.19 ± 1.78 [a, z, 2]	trt	<0.0001
	Rye	112.63 ± 1.79 [b, x, 2]	53.36 ± 2.12 [b, y, 2]	34.81 ± 1.79 [a, z, 2]	line	<0.0001
					day	<0.0001
10-20	Corn-Corn	546.4 ± 6.80 [a, x, 1]	384.93 ± 7.85 [a, y, 1]	116.15 ± 6.80 [a, z, 1]	trt*line	<0.0001
	Rye-Corn	534.40 ± 6.80 [a, x, 1]	248.87 ± 7.85 [b, y, 1]	98.35 ± 6.80 [ab, z, 1]	trt*day	<0.0001
	Rye-Rye	251.29 ± 7.37 [b, x, 1]	117.20 ± 6.80 [d, y, 1]	74.85 ± 6.80 [b, z, 1]	line*day	<0.0001
	Corn-Rye	278.50 ± 6.80 [b, x, 1]	178.62 ± 7.60 [c, y, 1]	95.70 ± 6.80 [ab, z, 1]	trt*line*day	<0.0001

Data is expressed as the LSmean ±SE. [a-c] Indicates significant differences between the treatments within the column at each time point. [x-z] Indicates significant difference between the genetic lines within the rows at each time point. [1-3]Indicates significant difference between the collection days within the columns at each time point.

Table 4. Evaluation of a nutritional rehabilitation model on bone parameters in three genetic chicken lines fed rye or corn at varying time points at day 20 of age.

Treatment/ variable	Genetic Line			Variable	P-Value
	Modern Broiler	1995 Line	Jungle Fowl		
Tibia Strength (kg/mm^2)					
Corn-Corn	3.90 ± 0.12 $^{a, y}$	3.47 ±0.14 $^{a, y}$	1.90 ± 0.15 $^{a, z}$	Trt	0.0001
Rye-Corn	3.73 ± 0.15 $^{a, y}$	2.18 ±0.16 $^{b, z}$	1.53 ± 0.15 $^{a, z}$	Line	0.0001
Rye-Rye	1.71 ± 0.14 $^{b, y}$	1.06 ±0.14 $^{c, z}$	1.28 ± 0.15 $^{a, yz}$	Trt * Line	0.0001
Corn-Rye	2.04 ± 0.13 $^{b, y}$	1.34 ± 0.14 $^{c, z}$	1.46 ± 0.15 $^{a, yz}$		
Tibia Ash (%)					
Corn-Corn	55.79 ± 0.79 $^{a, x}$	51.35 ± 0.88 $^{a, y}$	46.81 ± 0.95 $^{a, z}$	Trt	<0.0001
Rye-Corn	54.26 ± 0.88 $^{a, y}$	47.43 ± 1.02 $^{a, z}$	44.17 ± 0.95 $^{a, z}$	Line	<0.0001
Rye-Rye	45.76 ± 0.88 $^{b, y}$	37.19 ± 1.02 $^{b, z}$	42.31 ± 0.95 $^{a, y}$	Trt * Line	<0.0001
Corn-Rye	46.30 ± 0.83 $^{b, y}$	38.11 ± 0.88 $^{b, z}$	43.87 ± 0.80 $^{a, y}$		
Tibia Calcium (ppm)					
Corn-Corn	41.23 ± 0.37 $^{a, y}$	38.92 ± 0.42 $^{a, z}$	39.28 ±0.44 $^{a, z}$	Trt	<0.0001
Rye-Corn	41.94 ± 0.42 $^{a, y}$	38.24 ± 0.48 $^{ab, z}$	39.40 ± 0.44 $^{a, z}$	Line	<0.0001
Rye-Rye	39.13 ± 0.42 $^{b, z}$	37.80 ± 0.42 $^{ab, z}$	38.50 ± 0.44 $^{a, z}$	Trt * Line	0.0018
Corn-Rye	38.26 ± 0.39 $^{b, yz}$	36.42 ± 0.42 $^{b, z}$	39.05 ±0.44 $^{a, y}$		
Tibia Phosphorus (ppm)					
Corn-Corn	21.23 ± 0.26 $^{ab, y}$	20.20 ± 0.29 $^{a, yz}$	19.67± 0.31 $^{a, z}$	Trt	<.0001
Rye-Corn	21.69 ±0.29 $^{a, y}$	19.98 ± 0.33 $^{ab, z}$	20.00 ± 0.31 $^{a, z}$	Line	<.0001
Rye-Rye	20.36 ± 0.29 $^{ab, z}$	19.92 ± 0.29 $^{a, z}$	19.61 ± 0.31 $^{a, z}$	Trt * Line	0.0238
Corn-Rye	19.96 ± 0.27 $^{b, y}$	18.59 ± 0.29 $^{b, z}$	19.78 ± 0.31 $^{a, yz}$		

Data is expressed as the LSmean ±SE. $^{a-c}$ Indicates significant differences between the treatments within the column at each time point. $^{x-z}$ Indicates significant difference between the genetic lines within the rows at each time point.

Table 5. Evaluation of a nutritional rehabilitation model on morphometric analysis of duodenum in three genetic chicken lines fed rye or corn at varying time points at day 20 of age.

Treatment/ Variable	Genetic Line			Variable	P-Value
	Modern Broiler	1995 Line	Jungle Fowl		
Villus Height (µm)					
Corn-Corn	242.48 ± 15.94	244.57 ± 14.76	190.32 ± 17.47	Trt	0.0003
Rye-Corn	276.67 ± 15.94	250.22 ± 15.94	227.3 ± 17.47	Line	<0.0001
Rye-Rye	303.81 ± 15.94	313.13 ± 17.46	205.74 ± 15.94	Trt * Line	0.059
Corn-Rye	329.37 ± 15.94	324.17 ± 15.94	200.06 ± 19.53		
Crypt Depth (µm)					
Corn-Corn	30.47 ± 1.84 $^{b, z}$	24.41 ± 1.71 $^{c ,z}$	22.77 ± 2.02 $^{a, z}$	Trt	<0.0001
Rye-Corn	29.69 ± 1.84 $^{b, z}$	29.70 ± 1.84 $^{bc, z}$	24.18 ± 2.02 $^{a, z}$	Line	<0.0001
Rye-Rye	39.90 ± 1.84 $^{a, y}$	42.90 ± 2.02 $^{a, y}$	27.17 ± 1.84 $^{a, z}$	Trt * Line	0.0014
Corn-Rye	41.64 ±1.84 $^{a, y}$	37.63 ± 1.84 $^{b, y}$	23.08 ± 2.26 $^{a, z}$		
VH: CD (µm)					
Corn-Corn	7.96 ± 1.21	10.03 ± 1.12	8.56 ± 1.32	Trt	0.9001
Rye-Corn	9.57 ± 1.21	8.49 ± 1.21	9.44 ± 1.32	Line	0.1661
Rye-Rye	7.61 ± 1.21	7.38 ± 1.32	13.04 ± 1.21	Trt * Line	0.0886
Corn-Rye	8.04 ± 1.21	8.94 ± 1.21	8.88 ± 1.48		
Muscularis (µm)					
Corn-Corn	33.77 ± 2.87	33.58 ± 2.66	29.44 ± 3.15	Trt	0.203
Rye-Corn	30.12 ± 2.87	31.52 ± 2.87	24.70 ± 3.15	Line	0.0391
Rye-Rye	35.59 ± 2.87	29.59 ± 3.15	36.68 ± 2.87	Trt * Line	0.0609
Corn-Rye	35.03 ± 2.87	37.63 ± 2.87	22.91 ± 3.52		

Data is expressed as the LSmean ±SE. $^{a-c}$ Indicates significant differences between the treatments within the column at each time point. $^{x-z}$ Indicates significant difference between the genetic lines within the rows at each time point.

Table 6. Evaluation of a nutritional rehabilitation model on morphometric analysis of ileum in three genetic chicken lines fed rye or corn at varying time points at day 20 of age.

Treatment/ Variable	Genetic Line			Variable	P-Value
	Modern Broiler	1995 Line	Jungle Fowl		
Villus Height (µm)					
Corn-Corn	150.90 ± 11.49	181.8 ± 10.64	136.10 ± 12.59	Trt	0.3338
Rye-Corn	193.99 ± 11.49	171.58 ± 11.49	156.2 ± 12.59	Line	0.0026
Rye-Rye	165.61 ± 11.49	164.28 ± 11.49	142.55 ± 11.49	Trt * Line	0.048
Corn-Rye	190.36 ± 11.49	144.07 ± 11.49	141.56 ± 11.49		
Crypt Depth (µm)					
Corn-Corn	25.84 ± 1.79	26.78 ± 1.66	17.61 ± 1.96	Trt	0.1083
Rye-Corn	30.30 ± 1.79	26.36 ± 1.78	22.04 ± 1.96	Line	<.0001
Rye-Rye	28.46 ± 1.79	24.75 ± 1.78	19.92 ± 1.79	Trt * Line	0.2953
Corn-Rye	28.961 ± 1.79	22.40 ± 1.78	19.91 ± 1.79		
VH: CD (µm)					
Corn-Corn	6.62 ± 0.39	8.66 ± 0.36	6.92 ± 0.43	Trt	<.0001
Rye-Corn	7.62 ± 0.39	7.54 ± 0.39	6.36 ± 0.43	Line	0.8452
Rye-Rye	4.52 ± 0.39	5.05 ± 0.39	5.30 ± 0.39	Trt * Line	0.4854
Corn-Rye	4.54 ± 0.39	4.75 ± 0.39	5.99 ±0.39		
Muscularis (µm)					
Corn-Corn	26.34 ± 2.05	26.39 ± 1.89	25.11 ± 2.24	Trt	0.3216
Rye-Corn	29.80 ± 2.05	26.50 ± 2.04	26.61 ± 2.24	Line	0.0112
Rye-Rye	28.67 ± 2.05	25.03 ± 2.04	25.03 ± 2.04	Trt * Line	0.0561
Corn-Rye	35.53 ± 2.05	25.65 ± 2.04	22.84 ± 2.04		

Data is expressed as the LSmean ±SE. No differences were detected after conducted Tukey's post-hoc test.

	Trt	Trt until day 10	Trt after day 10
1	Negative Control	Corn based diet	Corn based diet
2	Treatment	Rye based diet	Corn based diet
3	Positive control	Rye based diet	Rye based diet
4	Treatment	Corn based diet	Rye based diet

Figure 1: Dietary treatments and timeline.

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Assessment of a Nutritional Rehabilitation Model in two modern broilers and their Jungle fowl ancestor: Evaluation of gut barrier function

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Abstract

This article is the second in a series of manuscripts to evaluate nutritional rehabilitation in chickens as a model to study interventions in childhood malnutrition (Part 2: Intestinal permeability and tight junction gene and protein expression). The objective of this study was to determine the effects of a rye-based diet during either the early or late phase of development, on paracellular intestinal leakage and tight junction (TJ) gene and protein expression across three diverse genetic backgrounds:Modern 2015 (MB2015) broiler chicken, random bred line initiated from 1995 (RB1995), and the Giant Jungle fowl (JF). Chickens were randomly allocated to four different dietary treatments. Dietary treatments were 1) a control corn-based diet throughout the trial (corn-corn [C-C]); 2) an early phase malnutrition diet where chicks received a rye-based diet for 10 days, and then switched to the control diet (rye-corn [R-C]); 3) a malnutrition rye-diet that was fed throughout the trial (rye-rye [R-R]); and 4) a late phase malnutrition diet where chicks received the control diet for 10 days, and then switched to the rye diet for the last phase (corn-rye C-R). Paracellular intestinal leakage was evaluated using fluorescein isothiocyanate dextran (FITC-D) and liver bacterial translocation. MB2015 and RB1995 consuming the rye-based diet showed a reduction in gut barrier function when compared to the corn fed chickens ($P < 0.05$). JF chickens that received the C-R diets showed a significant increase in duodenal ZO-2 gene expression when compared to other treatment groups, and when compared to both broiler lines. Interestingly, MB2015 chickens that received the C-C, R-C or R-R diets, had a significant increase in claudin-1 gene expression when compared to the MB2015 receiving C-R diet and the RB1995 or JF chickens. Overall, MB2015 appeared to have higher enteric permeability than the JF. Further studies to evaluate microbiome and inflammatory markers in these chicken models are currently being evaluated.

Keywords: Nutritional rehabilitation, chicken lines, paracellular gut leakage, tight junctions,

Introduction

Globally, malnutrition is the cause of 53% of all childhood mortality (Guerrant et al., 2008). Food insecurity and nutrient deficiency are often thought to be the primary etiology of childhood malnutrition and stunting in developing worlds (Solomons et al., 1993). Various feed interventions, like protein and vitamin supplementation, only reduced stunting by 36%, making it clear that the etiology of stunting is multifactorial (ZqA et al., 2008). Enteric infection along with malnutrition results in a "vicious cycle" of impaired gut barrier function and malabsorption (Guerrant et al., 2008). This cycle typically begins with the combination of undernutrition and high pathogen load in the environment which disturbs gut barrier function (Guerrant et al., 2008) increasing inflammation and bacteria translocation (Guerrant et al., 2008; Korpe and Petri, 2012). Intestinal inflammation interferes with the absorption of nutrients and disrupts the gut barrier function, which perpetuates environmentally induced enteropathy or environmental enteropathy (EE) (Korpe and Petri, 2012). EE is a subclinical condition that occurs in people living in unsanitary conditions and is characterized by morphological changes in the small intestine, including blunted villi, crypt hyperplasia, immune suppression, increased lymphocytes, increased intestinal permeability and malabsorption (Korpe and Petri, 2012). Adults with EE that were removed from the particular enteropathic environment were able to fully recover, improve intestinal morphology and decrease intestinal permeability (Gearson, 1971; Korpe and Petri, 2012) Nevertheless, EE plays an essential role in stunting in infants in developing countries (Keusch et al., 2013). Stunting has long-term consequences affecting overall fitness, cognitive development and drug absorption and economic productivity in adulthood and increase incidence of metabolic disorders like diabetes (Guerrant et al., 2008; Dewey and Begum, 2011). Women, who were stunted during their childhood, often have higher incidence of obstructed labor, and have

offspring with an increased risk of neonatal mortality and become stunted (Dewey and Begum, 2011). Therefore it is evident that childhood stunting is multifactorial and interventions are required to prevent long-term consequences on both human health and the local economy.

The intestinal epithelial cells are responsible for nutrient digestion, absorption and a physical barrier (Suzuki, 2013). There are two primary pathways in which molecule can pass the intestinal wall. Transcellular pathway facilitates the transport and absorption of nutrients through the cell via transporters channels on the luminal or basement membrane (Suzuki, 2013). Paracellular pathways promote transportation between adjacent enterocytes via pore formation of tight junction proteins (Suzuki, 2013). Barrier function failure results in bacteria and molecules that induce inflammation entering the host and stimulating the immune system, leading to chronic intestinal and systemic inflammation (Suzuki, 2013; Chen et al., 2015)**.** Tight junctions (TJ) are a group of proteins responsible for regulating paracellular transport and are primarily composed of occludin, claudins, and zona occludin (ZO). Occludin is a transmembrane protein responsible for controlling tight junction function and intermembrane and paracellular diffusion of small molecules and was first discovered in chicks (Chen et al., 2006, 2015; Ulluwishewa et al., 2011). Claudins are considered the backbone of tight junction proteins whose has both barrier and pore properties depending on the isoform of claudin (Ulluwishewa et al., 2011). There are 27 claudin genes identified in mammals although not all mammals contain all these genes (Günzel and Yu, 2013). There are only four claudin genes that have been identified in chickens (Claudin-1, 3, 5 and 16) but are thought to have similar functions to mammalian claudins (Awad et al., 2017). ZO is a plaque protein that allows transmembrane tight junction proteins to interact with the cytoskeleton and the peri junctional actomyosin ring and are involved in TJ stabilization and regulation (Ulluwishewa et al., 2011; Chen et al., 2015). Avian species tend to rely heavily on paracellular

pathways for the absorption of hydrosoluble compounds like glucose and amino acid, as well as some cation ion absorption because of their small intestinal tract (McWhorter et al., 2009). Intracellular signaling can regulate tight junction structure which controls paracellular permeability (Anderson and Van Itallie, 2009). Many physiological and pathological factors, including pathogens, can influence TJ assembly and maintenance, all of which affect barrier function (Awad et al., 2017).

When assessing intestinal permeability, it is essential to focus on both, barrier structure and function. There are multiple ways to measure intestinal permeability both directly and indirectly. In humans, intestinal permeability is often measured using inert oligosaccharides molecules like lactulose/mannitol, sucrose, and sucralose, which are given to patients orally and then estimated in the urine (Bischoff, 2011). Indirectly, intestinal permeability is measured by looking at bacteria associated markers or biomarkers related to epithelial cell integrity in the plasma or serum (Bischoff et al., 2014). Histological approaches of biopsied tissue can also indirectly measure intestinal permeability which includes mucin analysis, tight junction and defensins expression or morphometric analysis (Bischoff et al., 2014). In poultry, there are four primary tools to measure intestinal permeability. Our lab has recently optimized the use of fluorescein isothiocyanate dextran (FITC-D), a 3-5kDa fluorescent marker gavaged to birds and then measured in the serum as an indicator of paracellular transport (Tellez et al., 2014, 2015; Vicuña et al., 2015; Baxter et al., 2017). Our lab has also utilized bacteria related markers to indirectly measure intestinal permeability by enumeration aerobic bacteria in the liver as an indicator in failed barrier function (Tellez et al., 2014, 2015). Tight junction gene and protein expression can also be used to measure intestinal permeability in poultry (Awad et al., 2017). Lastly, Ussing chambers can be used to measure intestinal integrity as well as ion transport in both, human and animal studies (Bischoff et

al., 2014). This study utilized three of these methods to assess intestinal barrier structure and function in three different genetic lines of chickens consuming corn or rye-based diet. In a recent manuscript, our laboratories have shown that a rye-based diet, which is high in NSPs had malabsorptive effects on broilers, especially in the 1995 broiler line suggesting that chickens can be used as a model to study nutritional rehabilitation in human trials (Baxter et al., 2018). To further support the notion that chickens are a relevant model to study interventions on children with EE, intestinal permeability and structure must be assessed. Therefore, the current study had two primary objectives: To evaluate the effect of a rye-based diet on gut barrier dysfunction in three different genetic lines of chickens and to determine if the disturbance in gut barrier function could be repaired when the digestibility of the diet was improved.

Materials and Methods

Animals, diets and experimental design

All animal procedures were approved and in compliance with Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville (protocol #15006). Animal source, diet composition, and experimental design have previously been described by Baxter et al., (2018). In brief, three lines of chickens included in this study. For the modern broiler chickens (MB2015), one hundred and sixty one-day-old mixed broiler chicks, Cobb-Vantress, (Silom Springs, AR, USA) were used (n = 40 chickens/group). For the 1995 broiler chickens, one hundred and twelve one-day-old mixed broiler chicks, from the random-bred line initiated from 1995 (RB1995) Cobb broiler chicken line (Harford et al., 2014) were used (n = 28 chickens/group). And for the Jungle fowl chickens (JF), one hundred and sixty one-day-old mixed Giant Jungle fowl (Gyles et al., 1967) were used (n = 40 chickens/group). On the day of hatch, chicks were neck-tagged, weighed and randomly allocated to one of four dietary treatment groups in floor pens

containing new pine shavings in an environmentally controlled room. The primary energy source of each diet was either corn or rye plus soybean meal to meet the recommended requirements of a broiler starter diet according to the (NRC, 1994). Table 1 lists the diet composition and nutrient content for each experimental diet. Both diets were formulated to be isocaloric and isonitrogenous. When administering dietary treatment, the experiment was split into two phases, the first phase was from the day of hatch to D10 and the second phase was D10 to D20. Dietary treatments were 1) a control diet where chicks were maintained on a corn-based diet throughout the trial (corn-corn, C-C); 2) an early phase malnutrition diet where chickens were on a rye-based diet for 10 d, and then switched to the control diet (rye-corn, R-C); 3) a malnutrition rye-diet that was fed throughout the trial (rye-rye, R-R); and 4) a late phase malnutrition diet where chicks received the control diet for 10 d, and then switched the rye diet for the last phase (corn-rye, C-R). Individual body weights were recorded at day 9 and 19 to determine appropriate dosing for FITC-D. Chickens were euthanized via carbon dioxide asphyxiation and blood, liver and intestinal samples collected. The liver was collected aseptically to determine bacterial translocation, and blood samples were collected to measure Fluorescein isothiocyanate dextran (FITC-D) in the serum from all three genetic lines at D10 and D20. At D20, duodenum and ileal samples were collected for tight junction and inflammatory marker gene and protein expression from all three genetic lines.

Bacterial translocation and FITC-D

Briefly, approximately half the liver was aseptically removed from each chicken, collected into sterile bags, homogenized and weighed. Samples were then diluted 1:4 based on tissue weight with sterile 0.9% saline. Liver samples (n= 12)/chicken line) were then transferred to sterile 96 well Bacti flat bottom plate and diluted tenfold before being plated on TSA for evaluation of bacterial translocation. Samples were incubated under aerobic conditions at 37^{o} C for 24 h. Bacterial

translocation was expressed in colony forming units per gram of tissue (Log_{10} cfu/g). The FITC-D protocol used has previously been described by Baxter et al., (2017). In brief, chicks were orally gavaged with FITC-D (8.32 mg/kg) and 1h before sample collection, blood was collected from the femoral vein (n= 14) after chicks were euthanized. Serum was removed via centrifugation and diluted 1:5 in sterile 0.9% saline in 96 well flat bottom black plate. FITC-D was measured at excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA). Serum fluorescent concentrations were then determined using a standard curve and sera of chicks not given FITC-D.

RNA isolation, reverse transcription, and real-time quantitative PCR

Reverse transcription and quantitative PCR were performed as we previously described (Solomons et al., 1993; Lassiter et al., 2014). In brief, total RNA was extracted from gut tissues (n= 4/ chicken line) using Trizol reagent (Life Technologies, Grand Island, NY, USA) following manufacturer's guidelines. After verification of quality, integrity, and quantification by the Synergy HT multimode microplate reader (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA) and agarose gel electrophoresis, RNAs (1 µg) were reverse transcribed using qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD). RT products were then amplified using real-time quantitative PCR thermal cycler (qRT-PCR, Applied Biosystems 7500 Real-Time PCR system) with Power SYBR green master mix (Life Technologies, Grand Island, NY, USA). Primers specific for chicken Occludin, claudin-1, and ZO-2 were selected and displayed in Table 2. Ribosomal 18S was used as the reference gene (Table 2). The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). After the product was amplified, a melt curve analysis

was performed to determine nonspecific PCR products. Relative expression of target genes was determined by the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008; Hu et al., 2015).

Protein Extraction and Western Blot Analysis

Protein was isolated and quantified using methods previously described by (Solomons et al., 1993; Mussini, 2012; Lassiter et al., 2014). In brief, intestinal tissue (n= 4/chicken line) was homogenized in lysis buffer and quantified using Bradford assay kit (Bio-Rad, CA, USA) with the Synergy HT multimode microplate reader (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA). Proteins were then separated using a 4-12% Novex Bis-tris wedge-well gel (Invitrogen). A pre-stained molecular weight marker (Precision Plus Protein Dual Color Standard, Bio-Rad, CA, USA) was loaded onto the gel and used as a standard. The protein embedded gel was transferred onto a PDVF membrane (Immun-Blot PVDF Membranes for Protein Blotting, Bio-Rad, CA, USA) and blocked for an hour in 5% blocking buffer at room temperature and incubated with primary antibodies overnight at 4°C. The secondary antibody was applied to the membrane and incubated for one hour at room temperature. The primary and secondary antibodies applied to the membrane are displayed in Table 3. Protein loading was normalized to GAPDH. Before the membrane was developed and captured using the FluorChem M MultiFluor System (Proteinsimple, Santa Clara, CA, USA), chemiluminescence was applied to the membrane to enhance the signal (ECL plus) (GE Healthcare, Buckinghamshire, UK). Image Acquisition and Analysis were performed by AlphaView software (Version 3.4.0.0, 1993–2011, Proteinsimple).

Statistical analysis

Data were analyzed using a linear mixed model procedure in SAS (PROC MIXED; SAS Institute Inc., 2002) as previously described by Baxter et al., (2018). In brief, the factors of dietary treatment, genetic line, and the interaction between the dietary treatment and genetic line were fit as fixed effects for all variables. Significance was determined if $P < 0.05$. When factors were determined to be significant, pairwise comparisons were performed in SAS using the LSMEANS statement and corrected for multiple tests using Tukey post-hoc adjustment.

Results

The results of the evaluation of a nutritional rehabilitation model on serum FITC-D in three genetic chicken lines fed rye or corn at varying time points are summarized in Table 4. At ten days of age, chickens that received a rye-based diet in both the MB2015 and the RB1995 genetic lines showed a significant increase in FITC-D leakage when compared with the chicks that received the corn based diet. However, diet did not affect gut permeability in JF chickens. Interestingly and regardless of the dietary treatment, serum FITC-D levels varied significantly within genetic lines withMB2015 had the highest levels of serum FITC-D, followed by RB1995, and JF had the lowest levels (Table 4). At twenty day of age, chicks in the R-C treatment group had the same level of serum FITC-D as those maintained on a rye based diet in both MB2015 and RB1995 lines. Also, the C-R treatment group had a significantly higher level of serum FITC-D than the R-C group. However, chickens maintained on the corn based diet had a significantly lower serum FITC-D than those chicks on the R-C treatment group. This phenomenon was observed in both broiler genetic lines (MB2015 and RB1995). In contrast, there were no significant differences observed in gut barrier permeability in JF chickens regardless of the dietary treatment. Another intriguing result was that JF chickens that received a corn-based diet, for 20 days, showed significantly higher

levels of FITC-D when compared with the MB2015 and RB1995. Nevertheless, the opposite effect was observed in MB2015 and RB1995 chickens' in the C-R treatment group, which had significantly higher levels of serum levels of FITC-D when compared with JF chickens. No significant interaction between genetic lines was observed in chickens in the R-R and R-C treatment groups. Regardless of genetic line, treatment had a significant effect on gut leakage where corn-fed chicks had significantly lower serum FITC-D than those chicks fed rye at any phase of the experiment. Genetic line also had a significant effect on gut leakage where modern broiler had significantly higher serum FITC-D levels than 1995 broilers and JF (Table 4).

Table 5 shows the results of the evaluation of a nutritional rehabilitation model on liver bacterial translocation in three genetic chicken lines fed rye or corn at varying time points. At ten days of age, chickens from the MB2015 and RB1995 on the corn based diet had a significant increase in liver bacterial translocation when compared with the JF line. However, there were no significant differences between dietary treatments within each of the genetic lines (Table 5). However, regardless of genetic lines, rye fed chicks tended to have higher liver bacterial translocation than corn fed chicks. At twenty days of age, MB2015 and RB1995 chickens consuming a rye-based diet in the second phase of the experiment had higher liver BT compared to those chicks fed a corn based diet. Similar to what was observed in the serum FITC-D levels, the C-R treatment group had a significantly higher liver BT than the R-C group in both MB2015 and RB1995 chickens. In contrast, there was no significant differences between dietary treatments in JF. For chickens in the R-R treatment group, MB2015 had significantly higher liver BT than the JF (Table 5). Regardless of genetic line, treatment had a significant effect on hepatic bacterial translocation, chicks fed corn in the second phase of the experiment had significantly lower liver BT than the

chickens fed rye based diet. Regardless of dietary treatment, 1995 broiler had significantly more liver BT than the jungle fowl.

The results of the gene and protein expression of tight junctions in the duodenum of three different genetic lines of chickens consuming four different diets at twenty days of age are shown in Figure 1. There were no significant differences observed between dietary treatments, genetic lines, and the interaction between the dietary treatment and genetic line for occludin gene and protein expression for occludin and claudin-5 (Figure 1-A, 1-B and 1-C). Interestingly, JF chickens that received the C-R diet, have a significant increase in duodenal ZO-2 gene expression when compared to the other dietary treatments. In the corn-rye treatment group, JF had a significantly higher ZO-2 expression than the MB2015 and RB1995. However, JF chickens that received the R-R diet had a significant reduction in ZO-2 gene expression in the when compared to the MB2015 and RB1995 (Figure 1-A).

Figure 2 displays the results of the gene and protein expression of tight junctions in the ileum of three different genetic lines of chickens consuming four different diets at twenty days of age. No significant differences between dietary treatment, genetic line, and the interaction between the dietary treatment and genetic line were observed for occludin gene expression or occludin, claudin-1, claudin-5 or ZO-2 protein expression (Figures 2-A, 2-B and 2-C). However, MB2015 chickens that received the C-C, R-C or R-R diets, showed a significant increase in claudin -1 gene expression when compared to the MB2015 receiving C-R diet or the RB1995 or Jungle fowl chickens (Figure 2-A).

Discussion

Childhood malnutrition and stunting associated with intestinal barrier dysfunction and chronic inflammation are major contributors to child morbidity in low-income countries (Donowitz et al., 2016). Potential etiologies of these pathologies include high pathogen load and micronutrient deficiencies caused by the quality of the diet (Müller and Krawinkel, 2005). Chickens consuming high NSP diets also have poor growth and increased intestinal permeability (Tellez et al., 2014, 2015; Vicuña et al., 2015). This suggests that chickens can be utilized as a model to study the intestinal permeability and inflammation in children subjected to malnutrition and malabsorption. In the present study, three different genetic lines of chickens were treated with four different diets to induce early or late phase malnutrition. Modern broilers in the R-C group were able to reduce bacterial translocation however, their gut was still leaky when compared to the C-C treatment group. Modern broilers also appeared to have higher enteric permeability than the Jungle fowl chickens. Previous studies published by our laboratories, have shown that broilers and turkeys fed a rye-based diet have increased paracellular gut leakage measured via FITC-D and liver bacterial translocation (Tellez et al., 2014, 2015; Vicuña et al., 2015). The mucosal damage evoked by a rye-based diet has been associated to increase intestinal viscosity and dysbiosis within the intestinal tract (Tellez et al., 2014, 2015), but these detrimental effects can be attenuated when a rye-based diet is supplemented with a direct fed microbial that was selected for exogenous enzyme production (Latorre et al., 2015, 2016). The soluble non-starch polysaccharides (NSPs) in a rye-based diet increase digesta viscosity interfering with digestion and absorption of nutrients. Poultry has limited endogenous enzymes production that can hydrolyze NSPs (Vieira et al., 2014) which prevents nutrient digestion and absorption but can also lead to dysbiosis within the lower intestinal tract (Apajalahti and Vienola, 2016). The highly viscous digesta might also affect the osmotic

pressure within the intestinal tract, affecting ion transport and gut barrier function (Blikslager et al., 2007; Cronje, 2007). Therefore, it was expected that modern broiler genetic lines fed a rye-based diet would have higher levels of serum FITC-D and liver BT than corn-fed chicks at both D10 and D20. Interestingly, in both modern broiler lines, chickens in the group R-C showed an improvement in gut barrier function when compared with R-R or C-R chickens. In a recent study, we observed that chickens from the MB2015 line that received a rye-based diet for ten days and then switched for a corn-based diet (R-C), fully recovered body weight and bone mineralization when compared with R-R or C-R groups (Baxter et al., 2018). This may suggest that the intestinal epithelium recovered some barrier function or perhaps there was a balance in the dysbiosis described previously (Tellez et al., 2014). However, the intestinal barrier is still "leaky," and it is unclear if this is permanent or just has not been recovered within the observed time frame in this study. To determine a potential hypothesis as to why this occurred, it is important to review the mechanisms of enteric epithelium healing after intestinal injury. There are three primary events that occur after a mucosal injury that help restore intestinal barrier function. First, the intestinal villi contract to reduce the surface area which limits the area of repair and loss of barrier function during the repair (Blikslager et al., 2007). Interestingly, our previous reports indicate that the rye fed birds had significantly deeper crypts and longer villi than the corn-fed birds in the duodenum (Baxter et al., 2018). Therefore, this may suggest that rye fed chicks did not exhibit villi contraction typically seen after mucosal injury which may have been the reason why the C-R groups were not able to recover paracellular leakage. Second, the enterocytes nearby migrate to the area of the epithelial defect and undergo morphological changes in an attempt to seal the exposed basement membrane (Blikslager et al., 2007). These enterocytes then begin anchor themselves to the extracellular matrix (Blikslager et al., 2007). Lastly, enterocytes initiate tight junction expression

and reassembly, which is required to reduce the intercellular space between enterocytes. Therefore, the C-R groups may have had a leakier gut due to the higher intercellular space between epithelial cells which may not have been repaired within the observed time frame (Blikslager et al., 2007). Furthermore, the luminal accumulation of sodium and chloride ions can change the osmotic pressure within the lumen causing extracellular fluid from the paracellular space which reduces intercellular space (Blikslager et al., 2007). Hence, the change in diet may have affected the osmotic pressure of the lumen preventing the reduction in intercellular space of the R-C treated groups, allowing for more paracellular intestinal leakage.

In poultry, rye-based diets increase digesta viscosity and retention time, which facilitates bacterial colonization (Yegani and Korver, 2008). *Clostridium perfringens* is an ubiquitous opportunistic pathogenic bacteria commonly present in the microbiota of both humans and poultry (Yegani and Korver, 2008; Suzuki, 2013; Pan and Yu, 2014). *C. perfringens* produces several enterotoxins, which directly binds to the extracellular domains of claudin 3 and 4 (Sonoda et al., 1999). This stimulates internalization of these tight junction proteins, disturbing barrier function (Sonoda et al., 1999; Suzuki, 2013). Rye based diets can exacerbate levels of *C. perfringens* in poultry (Yegani and Korver, 2008). Human patients with EE and poultry consuming a rye-based diet have both been reported to contain pathogenic bacteria that can increase intestinal permeability (Korpe and Petri, 2012; Keusch et al., 2013; Latorre et al., 2015; Donowitz et al., 2016). This may suggest that the R-C groups in both the MB2015 and RB1995 broiler may still contain a higher number of pathogenic bacteria in the intestinal tract that may be contributing the increase in paracellular leakage. However, this hypothesis does not explain why the JF chickens showed better gut barrier function. Similar *to C. perfringens, E. coli* is a pathogenic bacteria that can disrupt intestinal tight junction barrier in humans (Suzuki, 2013; Awad et al., 2017) and are often a reservoir in poultry

(Bergeron et al., 2012). There are two serotypes *of E. coli* responsible for disrupting barrier function, Enteropathogenic *E. Coli* and Enterohemorrhagic *E. Coli* (Suzuki, 2013). Enteropathogenic *E. Coli* attaches to enterocytes causing cytoskeleton rearrangements and structural changes in the tight junction proteins and loss of barrier function (Muza-Moons et al., 2004). Enterohemorrhagic *E. coli* produces a Shiga toxin, inhibits cell protein synthesis which redistributes the tight junction proteins causing hyper permeability (Muza-Moons et al., 2004). *Vibrio cholera*, common contaminates of aquatic sources, produces two toxins, zonula occludins toxin and hemagglutinin/proteases (Vieira et al., 2014). Zonula occludin toxin disrupts the interaction between intracellular junctions (Zo-1) intercellular junctions (claudin and occludin) causing the permanent opening of tight junction proteins (Goldblum et al., 2011; Korpe and Petri, 2012; Vieira et al., 2014). Hemagglutinin/proteases increase intestinal permeability by cleaving the extracellular domain of occludin, disrupting intestinal barrier function.

Establishing commensal gut microbial community in broiler chicks during the early period post hatch is essential in controlling pathogenic infections in poultry. The small intestine microbiota is established within approximately two weeks of age, it takes the ceca much longer to establish microbial communities (6-7 weeks) (Lan et al., 2005). A similar trend was observed by Vieira et al., (2014) on children from the Gambia region of West Africa. They found that children suffering from persistent diarrhea with malnutrition could recover their body weight and have normal stool samples if children were treated for enteric infections along with intensive nutritional support (Vieira et al., 2014). However, this treatment was insufficient to repair intestinal structure and function or reduce immune stimulation (Vieira et al., 2014). They attributed the lack of small intestine repair to the antibiotic evading pathogenic bacteria within the intestinal tract as well as persistent antigens within the environment (Vieira et al., 2014). This again further supports the use

of chickens as a model to study childhood EE and malnutrition. Damaged mucosal barriers are unable to prevent foreign antigens from entering the circulatory system and lead to the activation of immune system and inflammatory response (Korpe and Petri, 2012). During an inflammatory response, the release of pro-inflammatory cytokines as well as other factors perpetuates the failure in barrier function (Feldman et al., 2005). In humans, bacterial translocation can be quantified by measuring serum LPS concentrations (Korpe and Petri, 2012). Bacterial translocation can occur by one of two pathways, paracellular transport or transcellular transport via endocytosis and lipid raft membranes. Although intestinal inflammation was not directly measured in the current experiment, Chen et al., (2015) found that leaky gut birds had increase level of serum endotoxin suggesting that there is a failure in the barrier function allowing pathogenic bacteria to enter the host. In the current experiment, broilers fed rye in the second phase of the experiment had a higher amount of bacterial translocation to the liver. Therefore, it is likely that there was an increase in intestinal inflammation which could perpetuate the vicious cycle of intestinal inflammation and gut barrier function reduction. Teirlynck et al., (2009) observed that broilers consuming a rye/wheat-based diet had T-cell aggregates in the mucosa as an indicator of excessive immune stimulation. A study in children in Bangladesh found that small intestinal bacteria overgrowth is associated with intestinal inflammation, stunting and poor sanitation (Donowitz et al., 2016). Small intestine bacterial overgrowth likely contributes to intestinal inflammation and micronutrient malabsorption pathogenesis in EE (Donowitz et al., 2016). This suggests that chickens consuming a diet containing high amounts of NSPs and children with EE are experiencing intestinal inflammation which interferes with intestinal function. Initially, quantitative genetic selection practices in poultry emphasized growth rates and body weight which are highly heritable traits (Tavárez and Solis de los Santos, 2016). However, this initially led to an increased incidence of

metabolic disorders like ascites and skeletal abnormalities (Tavárez and Solis de los Santos, 2016). More recently, poultry breeding companies have selected for feed efficiency using residual feed intake as well as selected against some of these metabolic disorders (Tavárez and Solis de los Santos, 2016). With proper nutrition and management, this has allowed the broiler industry to utilize chickens with higher productivity and efficiency (Tavárez and Solis de los Santos, 2016). There is very little difference in energy digestion between MB2015 and RB1995. Therefore, Mussini, (2012), suggest that genetic selection has improved metabolic efficiency in broilers consuming high quality feeds. It is evident from the current experiment that when modern broilers are removed from high quality feeds there are negative effects on growth and overall intestinal permeability. This is further supported by the observation that MB2015 chickens in the C-C, R-C, and R-R treatment groups had significantly higher ileal claudin-1 gene expression than the JF and RB1995. At D10, serum FITC-D was significantly higher in the MB2015 line than JF. Furthermore, MB2015 in the rye-rye treatment groups also had significantly higher levels of BT than the JF. Serum FITC-D and BT measure paracellular transport, a pathway avian species rely on for the absorption of hydro soluble compounds like glucose and amino acid, as well as some cation ion absorptions (McWhorter et al., 2009). This is likely an evolutionary adaptation to be able to consume diverse diets and is a mechanism to overcome the smaller intestinal surface area and shorter digesta retention time in birds (McWhorter et al., 2009). Genetic selection for growth in broiler chicks has led to the decreased relative size of the intestine but increased the intestinal surface area to increased villus size (Lilburn and Loeffler, 2015; Tallentire et al., 2016). Since birds rely on paracellular nutrient transport and broilers tend to have an increase in intestinal mass and surface area, this may indicate why modern broilers tended to have higher amounts of serum FITC-D and BT than the JF. However, barrier function assays did not correlate with tight junction

gene and protein expression. MB2015 on the C-C, R-C, and R-R diets had higher ileal claudin-1 mRNA expression. Claudin-1 is known to have pore sealing effects and increase expression of the protein lead to a decrease in permeability (Awad et al., 2017). Occludin expression has also been correlated with improved barrier function (Awad et al., 2017). However, McWhorter et al., (2009) found that occludin expressing cells had elevated transepithelial resistance (TER) but the same mannitol flux. This suggests that occludin sealing function depends on its attachment to ZO-1 and dissociation causes the tight junction to act as a pore (McCarthy et al., 1996).Therefore it is unclear why the MB2015 chickens had functionally leakier guts and lower intestinal integrity, yet higher expression of the genes and proteins regulating permeability. This may suggest that modern broilers consuming a digestible diet have less selective paracellular nutrient transport, which may be an adaptation to their attenuated acute phase immune response (Leshchinsky and Klasing, 2001). Treatment did not have a significant effect on occludin or claudin-1 mRNA expression in both the duodenum and the ileum. Similar results were observed in mRNA expression of occludin, claudin-1 or ZO-1 between broilers consuming a corn-based diet or a rye-wheat-barley grower diet and challenged with coccidiosis (Chen et al., 2015). Osselaere et al., (2013) also found that in the presence of mycotoxins there was no difference in claudin-5, claudin-1 and ZO-1 and ZO-2 mRNA expression in the duodenum. However, supplementing the diet with mycotoxin absorbent lead to an increase in claudin-5, claudin-1, ZO-1 and ZO-2 mRNA expression in the ileum. This may suggest that the particular mRNA expression of various tight junctions is dependent on the tissue and certain stimuli (Osselaere et al., 2013). There have also been reports in poultry that LPS decreased occludin and ZO-1 and Muc2 gene expression (Lee et al., 2017). There were no differences in jejenual claudin-5 and ZO-2 mRNA expression between broilers under thermoneutral conditions and broilers supplemented with 2.5% Galacto-oligosaccharides (GOS)

under heat stress conditions (Varasteh et al., 2015). Varasteh et al., (2015) suggested that the GOS is directly interacting with immune and epithelia cells to improve intestinal integrity. Therefore, it is evident from the current and previous studies, that carbohydrate sources can affect tight junction gene expression in broilers chickens (Osselaere et al., 2013; Varasteh et al., 2015). Duodenal claudin-1 gene expression was measured but not displayed in Figure 1 due to the low detection level in the JF. This maybe a function of age as JF take longer to mature than broilers (Wall and Anthony, 1995). This may also indicate the genetic selection for growth has changed the distribution of tight junction in the intestinal tract, as the JF have had no selection pressure for growth. In rodents, claudin-1 expression is the lowest in the duodenum and has the highest level of expression in the colon (Markov et al., 2010). However, Lu et al., (2013) found contrasting results in rats where the duodenum and colon have the highest expression of claudin-1 because theses membranes are less permeable. Grilli et al., (2016) observed a downregulation of claudin-1 gene expression all along the gastrointestinal tract and an upregulation of proinflammatory cytokines gene expression in the upper intestinal tract in weaned piglets treated with butyrate. Therefore the level of claudin-1 gene expression may be lower in the duodenum of JF due to the higher amount of inflammation in all treatment groups because they are not adapted to either diet. Nevertheless, contrasting results have been documented in humans, where patients with inflammatory bowel disease had higher mRNA expression of claudin-1 (Turner, 2009). Therefore, it is evident that both modern chicken lines consuming rye-based diet had similar intestinal lesions and permeability to patients with EE, making it a relevant animal model to determine interventions. In broilers, nutrition alone could not fully repair the gut leakage within the observed time frame, which may have been due intestinal dysbiosis, reduce barrier function or indirectly inflammation. Therefore, in order to restore barrier function and increase nutrient absorption it may be important

to reduce pathogen load and immune stimulation. This makes it essential to find interventions that are not only able to increase nutrient availability but reduce pathogenic bacterial colonization. Further studies to evaluate microbiome and inflammatory markers in these chicken models are currently being evaluated.

Table 1. Composition and Nutrient Content of the experimental diets (%)

Item Ingredients (%)	Rye-based diet	Corn-based Diet
Corn	-	57.32
Rye	58.27	-
Soybean meal	31.16	34.66
Poultry fat	6.30	3.45
Dicalcium phosphate	1.80	1.86
Calcium carbonate[1]	1.10	0.99
Salt	0.38	0.38
DL-Methionine	0.35	0.33
Vitamin premix[1]	0.10	0.20
L-Lysine HCl	0.22	0.31
Choline chloride 60%	0.10	0.20
Mineral premix[2]	0.12	0.12
Threonine	0.08	0.16
Antioxidant[3]	0.02	0.02
Calculated analysis		
Metabolizable energy (kcal/kg)	2850	3035
Crude protein, %	22.38	22.16
Lysine, %	1.32	1.35
Methionine, %	0.64	0.64
Methionine + Cystine, %	0.98	0.99
Threonine, %	0.86	0.91
Tryptophan, %	0.30	0.28
Total calcium, %	0.90	0.9
Available Phosphorus (%)	0.45	0.45
Sodium (%)	0.16	0.16

[1]Vitamin premix supplied the following per kg: vitamin A, 20,000 IU; vitamin D3, 6,000 IU; vitamin E, 75 IU; vitamin K3, 6.0 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 µg; and ascorbic acid, 200 mg (Nutra Blend LLC, Neosho, MO 64850).
[2]Mineral premix supplied the following per kg: manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg (Nutra Blend LLC, Neosho, MO 64850).
[3]Ethoxyquin.

Table 2. Oligonucleotides qPCR primers.

Gene	Accession Number	Primer Sequence	Orientation	Manufacturer	Product length (BP)
Occludin	NM_205128	CGCAGATGTCCAGCGGTTA	Forward	Thermo Fisher Scientific	60
		GTAAGGCCTGGCTGCACATG	Reverse		
Claudin-1	NM_001013611	CCCACGTTTTCCCCTGAAA	Forward	Integrated DNA Technologies	61
		GCCAGCCTCACCAGTGTTG	Reverse		
Zo-2	XM_015280247	GCAATTGTATCAGTGGGCACAA	Forward	Integrated DNA Technologies	69
		CTTAAAACCAGCTTCACGCAACT	Reverse		
18S	AF173612	TCCCCTCCCGTTACTTGGAT	Forward	Integrated DNA Technologies	60
		GCGCTCGTCGGCATGTA	Reverse		

Table 3. Primary Antibodies for Western Blot.

Antibody	Antibody Source	Dilution	Manufacturer	Protein Size (kDa)
Occludin	Rabbit	1:500	Santa Cruz Biotechnology	60-82
Claudin-1	Goat	1:500	Santa Cruz Biotechnology	22
Claudin-5	Rabbit	1:1000	Santa Cruz Biotechnology	23
ZO-2	Rabbit	1:500	Abcam	150
GAPDH	Rabbit	1:1000	Santa Cruz Biotechnology	37

Table 4. Evaluation of a nutritional rehabilitation model on serum FITC-D (ng/mL) in three genetic chicken lines fed rye or corn at varying time points.

Day of Evaluation	Treatment	Genetic Line		
		MB2015	RB1995	Jungle Fowl
Day 10	Corn	1162.83 ± 70.47 $^{b, x}$	166.32 ± 77.53 $^{b, y}$	8.64 ± 60.69 $^{a, z}$
	Rye	1743.53 ± 66.28 $^{a, x}$	352.62 ± 84.49 $^{a, y}$	7.21 ± 65.04 $^{a, z}$
Day 20	Corn-Corn	8.52 ± 32.83 $^{c, z}$	8.97 ± 43.43 $^{c, z}$	126.61 ± 32.83 $^{a, y}$
	Rye-Corn	158.32 ± 32.83 $^{b, z}$	231.38 ± 38.84 $^{b, z}$	184.27 ± 32.83 $^{a, z}$
	Rye-Rye	220.98 ± 38.84 $^{b, z}$	386.97 ± 38.84 $^{ab, z}$	265.77 ± 32.83 $^{a, z}$
	Corn-Rye	433.09 ± 34.07 $^{a, y}$	413.41 ± 37.04 $^{a, y}$	257.62 ± 32.83 $^{a, z}$

Data is expressed as the mean ± SE. ($P<0.05$)

$^{a-c}$ Indicates significant differences between the treatments within the column at each time point respectively.

$^{x-z}$ Indicates significant difference between the genetic lines within the rows at each time point respectively.

Table 5. Evaluation of a nutritional rehabilitation model on liver bacterial translocation (Log_{10} CFU/g) in three genetic chicken lines fed rye or corn at varying time points.

Day of evaluation	Treatment	Genetic Line		
		MB2015	RB1995	Jungle Fowl
Day 10	Corn	1.95 ± 0.36 [a, y]	1.77 ± 0.29 [a, y]	0.81 ± 0.29 [a , z]
	Rye	2.44 ± 0.36 [a, z]	2.11 ± 0.29 [a, z]	1.54 ± 0.29 [a, z]
Day 20	Corn-Corn	0.40 ± 0.32 [a, z]	0.89 ± 0.32 [a, z]	0.62 ± 0.33 [a, z]
	Rye-Corn	0.42 ± 0.32 [a, z]	1.11 ± 0.32 [a, z]	1.13 ± 0.32 [a, z]
	Rye-Rye	2.73 ± 0.35 [b, y]	2.36 ± 0.32 [b, yz]	0.88 ± 0.37 [a, z]
	Corn-Rye	2.05 ± 0.32 [b, z]	1.96 ± 0.32 [b, z]	1.09 ± 0.32 [a ,z]

Data is expressed as the mean ± SE. ($P<0.05$)

[a-c] Indicates significant differences between the treatments within the column at each time point respectively.

[x-z] Indicates significant difference between the genetic lines within the rows at each time point respectively.

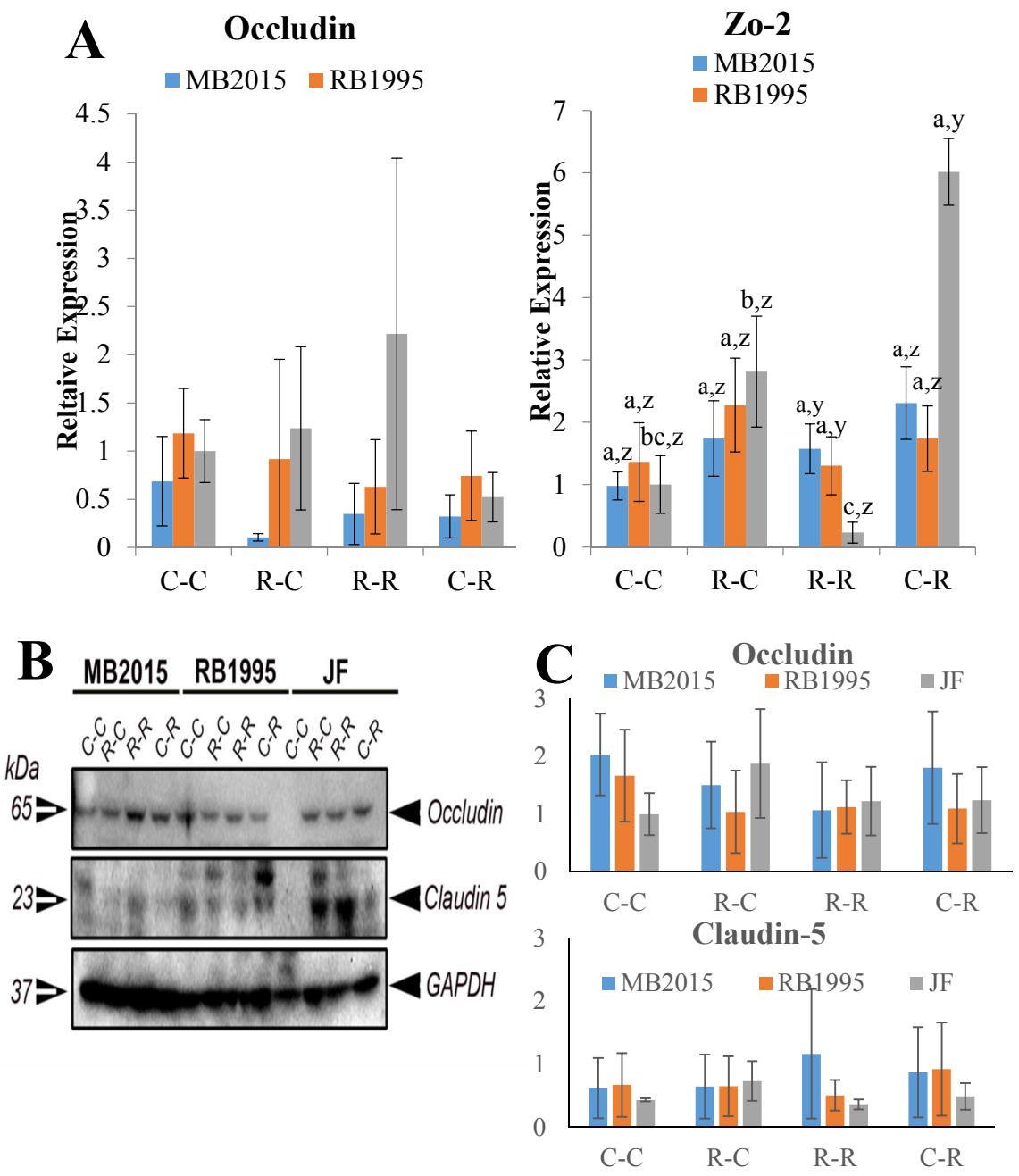

Figure 1: RT-qPCR results of tight junction mRNA expression (Occludin and ZO-2) in the duodenum are shown in (A). Data is expressed as fold change normalized to the JF C-C group. Superscripts are used to identify significant interaction between genetic line and treatment. [a-c] Indicates significant differences between the treatments at each time point. [y-z] Indicates significant difference between the genetic lines at each time point ($P < 0.05$). Western blot of occludin and claudin-5 in the duodenum of three different genetic lines of chickens consuming four different diets are displayed in (B). Relative protein expression for each of the tight junction proteins measured via western blot is displayed in (C) ($P > 0.05$).

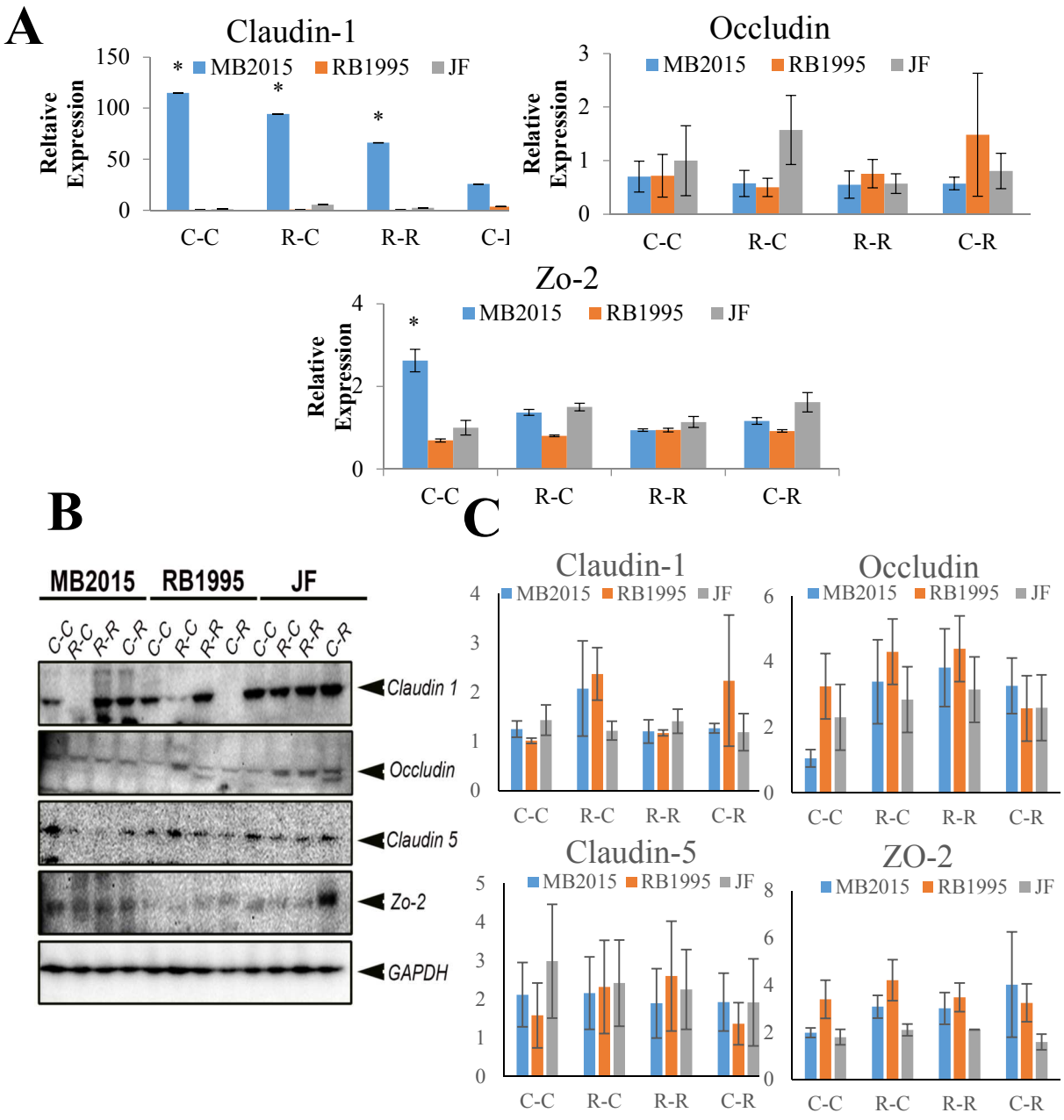

Figure 2: RT-qPCR results of tight junction mRNA expression (Claudin-1, Occludin and ZO-2) in the ileum are shown in (A). Data is expressed as fold change normalized to the JF C-C group. * Indicates significant difference between the genetic lines at each time point ($P < 0.05$). Western blot of Claudin-1, Occludin, Claudin-5, ZO-2 in the ileum of three different genetic lines of chickens consuming four different diets are displayed in (B). Relative protein expression for each of the tight junction proteins measured via western blot is displayed in (C) ($P > 0.05$).

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Conclusion

This studies presented in this dissertation aimed to determine if chickens fed a malabsorptive diets, using a rye based diet, could mimic the disease state of stunted children. From the literature it is evident that stunting is a global problem with multifactorial etiologies. The short term effects of stunting include increased risk of morbidity and mortality. There are also long-term consequences of stunting including like cognition, executive function and school attainment. One of the key observation in detecting stunting was increased intestinal permeability. Therefore, before utilizing chickens as a potential model to study childhood malnutrition, a reliable assay is required to measure intestinal permeability. FITC-D is a fluorescent marker commonly used to measure intestinal permeability in mice. Previous research in our laboratory developed this marker for chickens, however optimization was required to obtain reliable and consistent results. To induce intestinal inflammation, a 24hr feed restriction (FR) model was used as previously described by Vicuña et al., (2015). Optimization was attained by increasing the dose, reducing the collection time, reducing the gain value, using blank sera as a background and developing a standard curve with every assay. The combination of these methods reduced the amount of background fluoresce and increased the difference between control and FR groups so significant differences could be detected. This modification to the exiting assay offered a more reliable assay to measure intestinal permeability.

To determine if chicken would make a viable model to study child malnutrition, a rye based diet was used to induce malnutrition during an early or late phase of growth in three different genetic lines of chickens. Within each genetic line, growth, intestinal morphology, bone quality and intestinal permeability varied greatly between dietary treatments. There was no difference in BW and BWG between modern broilers in the rye–corn treatment group, early phase malabsorptive

diet, and those maintained a corn-based diet throughout the experiment. Also, modern broilers in the rye-corn and corn-corn treatment groups had significantly stronger tibias, higher amounts of tibia ash, and higher levels of calcium than broilers in the rye-rye and corn-rye treatment groups. Modern broilers in the rye-corn group had lower hepatic bacterial translocation and shallower duodenal CD when compared to those fed rye in the second phase of the experiment. However, the rye-corn treatment group had significantly higher serum FITC-D their when compared to the corn-corn treatment group. It is evident that modern broiler in the rye-corn treatment group exhibited catch up growth and was able to fully recover all of the growth and bone parameters measured after the consumption of a rye based diet. The exception in which the rye-corn group was unable to recover was the serum FITC-D indicating the gut was still leaky. The 1995 broiler in the rye–corn treatment group had a higher BW, BWG and tibia strength than those chicks fed rye in the second phase of the experiment. However, the rye-corn group weighed significantly less and had significantly weaker tibias than the corn–corn group. Also, CD in the rye-corn group was significantly deeper than the corn-corn treatment group but was shallower than the rye-rye treatment group. This suggests that BW, BW, tibia strength and CD was not fully recovered within the observed timeframe. However, the rye-corn group had the same percent of tibia ash as the corn-corn treatment group, which was higher than those chicks fed rye in the second phase of the experiment. This indicates that bone mineralization was repaired within the observed time frame. The 1995 broilers fed corn in the second phase of the experiment had a reduction in hepatic bacterial translocation but the rye-corn treatment group had significantly higher serum FITC-D when compared to the C-C treatment group. It is unknown if the “leaky” intestinal barrier observed in both the modern broilers and 1995 broilers is permanent or just has not been recovered within the observed time frame in this study. Also, the increased paracellular

leakage in the rye-corn treatment group may be due to a higher number of pathogenic bacteria in the intestinal tract. Jungle Fowl in the corn–corn group had significantly higher BW and BWG than the rye–rye group, likely due to the anti-nutritional factors in the rye-based diet. However there were no differences between any of the other treatment groups for bone quality and intestinal morphology. Intestinal permeability did not differ between dietary treatments for the jungle fowl. However, the corn-rye treatment group had significantly higher Zo-2 expression than the other dietary treatments suggesting dietary treatments affected intracellular tight junction gene expression. Jungle fowl appeared to have a higher tolerance to the rye based diet, as there were minimal differences between dietary treatments for the parameters measured.

There were drastic differences between the genetic lines for many of the parameters measure. Modern broilers had the highest BW and BWG while jungle fowl had the lowest. The difference in growth between the modern broiler and the 1995 broiler suggests that genetic selection within the last 20 years has allowed modern broilers to obtain higher body weight and recover more quickly after a period of undernutrition. Regardless of dietary treatment, modern broilers fed rye in any phase of the experiment had significantly deeper CD, longer villi, and thicker muscularis than the Jungle Fowl in both the duodenum and ileum. Modern broilers also appeared to have higher enteric permeability than the Jungle fowl chickens, yet overall had higher expression of the genes and proteins regulating permeability. Modern broilers may have less selective paracellular nutrient transport, which may reflect their attenuated innate immune response. JF chickens had better gut barrier function regardless of diet when compared to the commercial broiler lines. This maybe a function of diet tolerance as jungle fowl are not adapted for either diet.

It is evident from this experiment that rye reduced growth and increased intestinal permeability in chickens. This suggests that a rye-based diet is a viable approach to induce undernutrition in chickens to study compensatory growth and clinical interventions in malnourished children. The slower compensatory growth rate observed in the 1995 broilers consuming was similar to that of stunted children in developing countries. This suggests that the 1995 broilers would be the most viable to study nutritional rehabilitation in humans.

Reference

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Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Dr. Lisa Bielke

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee (IACUC)

DATE: September 8, 2014

SUBJECT: IACUC APPROVAL
Expiration date: September 14, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED protocol 15006: *Development of enteric inflammation models for investigation of antibiotic alternatives in poultry*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond September 14, 2017 you must submit a new protocol prior to that date. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

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