# A STUDY OF THE EFFICACY OF PROBIOTIC SUPPLEMENTATION TO REDUCE AGGRESSIVE FEATHER PECKING BEHAVIOR IN LAYING HENS

A Thesis

by

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# ABSTRACT

Feather pecking behavior poses a significant challenge within the egg production industry. A recent shift in housing structure and management style has led to an increased incidence of this destructive behavior which negatively effects animal welfare and economic returns. The pulling of feathers results in pain, injury and increased rates of mortality and cannibalism. It also compromises efficiency as chickens denuded by feather pecking require more feed to maintain body temperature resulting in decreased food conversion ratios. Although this deleterious behavior is present in all housing systems it is amplified in systems that congregate large numbers of hens together allowing for increased contact between conspecifics.

Many studies indicate that behavior can be influenced by the gut microbiome, especially during critical stages of early development. This is relevant to the egg industry because in an effort to limit bacterial pathogens, hens are hatched in "clean environments" excluding them from exposure to early beneficial microflora supplied by healthy adult animals. Hen development and early intestinal microbial colonization occur in concert providing a framework for host function and behavior throughout life.

The current study recognizes this relationship and explores the use of continuous flow (CF) and electron beam irradiated continuous flow (EB) cultures to encourage early intestinal colonization with the intent of reducing feather pecking behavior. These cultures were evaluated for their effectiveness using a chicken model. Three treatments; CF, EB and a treatment that contained only sterilized water (WR) were administered as weekly supplements to birds at day of hatch and throughout the first 16 weeks of life. Behavioral testing, physiological testing and

video observation of pen behaviors were used to identify any significant differences between treatments.

Of the results garnered from this study, very few significant differences were found between treatment groups. No difference was found in plasma corticosterone levels or hen performance during the tonic immobility test. One of the few significant differences identified through video observation of home pen behaviors was the number of drinks ($p = 0.02$) performed by hens in the WR treatment group. The WR treatment group also neared significance in the number of times hens were observed standing ($p = 0.07$) in the home pen and in latency to first step ($p = 0.09$) as evaluated using the open-field test. The number of gentle feather pecks delivered was also found to be significant in the WR group, however this result is likely biased due to the number of zeroes included in this data set and requires a larger study in order to confirm this observation. Overall this study was unable to identify any differences between treatment groups but provided evidence that suggests that further research is prudent. Ultimately, this work supports further exploration into the area of probiotic culture-based solutions for altering laying hen behavior in a commercial setting.

# DEDICATION

To my wonderful wife Amy and amazing daughters, Mazie and Georgia.

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## CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a thesis committee consisting of Professor Dr. Suresh Pillai of the Department of Food Science and Technology and Dr. Kenneth Genovese of the Department of Poultry Science and Professor Dr. Courtney Daigle of the Department of Animal Science and Professor Dr. Arundhati Rao of the Department of Pathology and Laboratory Medicine.

16s rRNA sequencing of the CF culture analyzed in Chapter IV was performed by MR DNA, Shallowater, TX, United States. Statistical analysis for this study was supervised by Dr. Shuling Liu and performed by Daniel Zilber, Junsouk Choi, Guanxun Li and Honggang Wang of the Department of Statistics. All other work conducted for the thesis was completed by the student independently.

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# NOMENCLATURE

| | |
|---|---|
| CF | Continuous Flow Culture |
| EB | Electron Beam Irradiated Continuous Flow Culture |
| CE | Competitive Exclusion Culture |
| Ebeam | Electron Beam |
| WR | Sterile Distilled Water |
| FP | Feather Pecking |
| GFP | Gentle Feather Pecking |
| SFP | Severe Feather Pecking |
| IFP | Injurious Feather Pecking |
| 5-HT | 5-hydroxytryptamine (Serotonin) |
| QTL | Quantitative Trait Loci |
| INF-γ | Interferon-γ |
| IDO | Indoleamine 2,3-dioxygenase |
| KYNA | Kynurenic Acid |
| PIC | Picolinic Acid |
| 3-HK | 3-Hydroxykynurenine |
| QUIN | Quinolinic Acid |
| NMDA | N-methyl-D-aspartate |
| DA | 3,4-dihydroxyphenethylamine (Dopamine Activity) |
| HPA | Hypothalamic Pituitary Adrenal |
| IL-1 | Interleukin-1 |

| | |
|---|---|
| IL-6 | Interleukin-6 |
| IL-10 | Interleukin-10 |
| TNF- α | Tumor Necrosis Factor α |
| CORT | Corticosterone |
| PVN | Paraventricular Nucleus |
| CRF | Corticotropin Releasing Factor |
| SNS | Sympathetic Branch of the Autonomic Nervous System |
| ACTH | Adrenocorticotropic Hormone |
| GABA | γ-aminobutyric Acid |
| GF | Germ Free |
| SPF | Specific Pathogen Free |
| BDNF | Brain-Derived Neurotrophic Factor |
| VN | Vagus Nerve |
| ENS | Enteric Nervous System |
| PRR | Pattern Recognition Receptors |
| SCFA | Short Chain Fatty Acids |
| BBB | Blood Brain Barrier |
| MIA | Maternal Immune Activation |
| RPCF | Recombined Porcine Derived Continuous Flow |
| PCF-1 | Porcine Derived Competitive Exclusion Culture |
| OTU | Operational Taxonomic Unit |
| TI | Tonic Immobility |
| AP | Aggressive Peck |

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| CFU | Colony Forming Units |
| USDA-ARS-FFSU | United States Department of Agriculture-Agricultural Research Unit-Food and Feed Safety Research Unit |

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# CHAPTER I

# INTRODUCTION

The dynamic relationship between gut microflora and host has developed over many millennia. Metazoans evolved in concert with microbial life and therefore over time established symbiotic interactions that place the intestinal microbiota at a central position within host health and disease (Sekirov, Russell, Antunes, & Finlay, 2010). Many beneficial elements of this relationship have been described, including assistance in immune and intestinal development, aiding in nutrient digestion, and prevention of pathogenic infection through competitive exclusion (Nisbet, Corrier, & Deloach, 1993, Marcolla, Alvarado, & Willing, 2019).

As global demand for safe and economical animal-based food products grow the livestock industry must optimize animal efficiency to meet it. To facilitate production efficiency many modern rearing practices include changes in diet, antimicrobial use and sanitation; all of which may inadvertently compromise early microbial gut colonization (Marcolla, Alvarado, & Willing, 2019). This can be illustrated in poultry production systems where laid eggs are rapidly collected, transferred to hatchery facilities, disinfected and artificially incubated (Marcolla, Alvarado, & Willing, 2019). Once hatched, chicks are then transported and housed in sanitized barns absent of adults. This practice reduces exposure to commensal organisms that co-evolved with the host and may lead to a phenomenon of "disappearing microbiota" which may have unintended consequences on animal development and future behavior (Blaser, 2017).

One of those unintended consequences may be aggressive feather pecking behavior in laying hens. Injurious feather pecking behavior is an economic and animal welfare issue within the egg production industry and solutions currently being utilized are associated with animal

welfare concerns of their own. New methods for reducing this unwanted behavior are needed and the relationship between early gut colonization and behavior may offer a solution. Feather pecking behavior is a complex problem that is influenced by multiple elements such as nutrition, social influence, genetics, environment and immunology. Many of these factors are either directly or indirectly influenced by the gut microbiome, making it a potential inherent tool for the reduction of this behavior.

Over the last thirty years scientist at the U.S. Department of Agriculture, Agricultural Research Service, Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center located in College Station, Texas have developed and maintained constant flow mixed bacterial cultures of cecal content sourced from avian and porcine origin. Primarily these cultures have been utilized as competitive exclusion (CE) cultures, delivered to early postnatal animals to effectively outcompete pathogenic bacteria for colonization of the gut (Nisbet, Corrier, & DeLoach, 1993; Nisbet, et al., 1994; Genovese, et al., 2003). Nisbet, Corrier, & DeLoach, 1993, provided evidence that these defined mixed CF cultures could be maintained in an *in vitro* CF culture system (chemostats) and that they retained their protective efficacy over time (Nisbet, Corrier, & DeLoach, 1993). This is significant because Stavric & D'aoust, 1993, found that mixed cultures of cecal microflora that were grown separately and mixed together directly before delivery were less protective than mixed cultures grown together (Stavric & D'aoust, 1993). Furthermore, it was shown that these CF cultures rapidly colonize the gut of the host when orally administered (Nisbet, et al., 1994; Genovese, et al., 2003). These attributes made the chemostat maintained CF culture an appropriate probiotic for use in experiments associated with early gut colonization.

Beyond its potential for maintaining a sustained CF culture, the chemostat allows for the same CF culture to be anaerobically sourced and packaged for electron beam (eBeam) processing, a procedure that prevents bacterial multiplication through direct and indirect DNA damage. EBeam technology utilizes ionizing radiation that targets nucleic acids without compromising bacterial cell wall integrity or surface macromolecules (Kogut, et al., 2012; Jesudhasan, et al., 2015). This is an important aspect of eBeam inactivated bacterial cultures because they retain their immunogenicity as well as metabolic activity after treatment (Kogut, et al., 2012; Hieke & Pillai, 2018) offering valuable insight as to the impact of a non-colonizing bacterial cultures with regard to behavior.

The overall hypothesis of this study is that early supplementation of CF and eBeam irradiated CF culture (EB) will reduce the number of aggressive feather pecks and increase the stress coping abilities of laying hens compared to non-supplemented hens. In this study we provided laying hens with a weekly supplementation of CF, EB or sterile distilled water (WR) for the first 16 weeks of life in order to evaluate changes across treatments in pecking behavior and stress coping ability through video observation, behavioral testing and corticosterone analysis.

The specific objectives of the research were:

(1) Identify the microbial profile of an established CF culture using 16S rRNA analysis.

(2) Evaluate the efficacy of the CF and EB culture to reduce aggressive feather pecking through video observation and analysis.

(3) Evaluate the efficacy of the CF and EB culture to increase stress coping abilities through behavioral assessments such as open field and tonic immobility testing.

(4) Assess the physiological response to stress through corticosterone testing.

# CHAPTER II

# LITERATURE REVIEW

## Laying Hen Housing Management

Over the last twenty years animal welfare concerns have driven a change in the egg production industry. These changes have been spurred by an expanding consumer base that demands information about how production food animals are managed and cared for (Mitchell, 2001). The area of greatest concern with regard to egg production deals with laying hen housing systems. Over the past ninety years in the United States and throughout most of the world conventional (battery) cage style systems have been used to house commercial laying hens (IEC, 2009). These housing systems became popular in the 1950s because they offered many advantages over previous housing styles. The main advantage being that it created a system that separated hens and eggs from fecal droppings, thereby, reducing the potential for pathogenic infection (Mench, Sumner, & Rosen-Molina, 2011). There were also economic benefits to these systems allowing for efficient egg collection, greater control of environmental variables, and automated feeding and watering (Mench, Sumner, & Rosen-Molina, 2011). Over time, the efficiency of these systems was maximized with the addition of larger cages in order to accommodate more birds, increasing stocking densities within a housing facility (Mench, Sumner, & Rosen-Molina, 2011). The modern egg production industry was developed using this type of housing system and according to the International Egg Commission in 2016, 89.7 percent were still utilizing them, producing the majority of 72 million metric tons of eggs (Conway, 2017).

While efficient, these conventional style systems started to draw criticism from public consumers in Europe around the 1960s (Mench, Sumner, & Rosen-Molina, 2011). Publications like Ruth Harrison's book, *Animal Machines* (Harrison, 1964) and the United Kingdom's Brambell Report (Brambell, 1966) raised public awareness to the topics of production animal health and welfare and would eventually influence future legislative policy like the European Union Council Directive 1999/74/EC that stipulated that laying hens in the EU could only be housed in either enriched or non-caged systems from 2012 onwards (Heerkens, et al., 2015; Mench, Sumner, & Rosen-Molina, 2011; EU, 1999). The welfare concern relating to conventional cage style housing systems revolved around limitations on physical space for the laying hens. These limitations on space restrict the hen's ability to perform natural behaviors like dust bathing, running, wing flapping, flying, nesting, and perching (Leyendecker et al., 2005, as cited in Heerkens, et al., 2015). As a result, Non-caged and enriched caged housing systems were developed in an effort to provide laying hens with opportunities to engage in biological appropriate physical behaviors.

There are two main design types for non-cage housing systems, multi-tier aviaries and single level littered floor systems (Hester, 2014). Both allow hens to move freely within a littered floor area, providing the environment necessary to perform dust bathing, scratching and foraging (Hester, 2014). Enriched caged systems still utilize cages but incorporate perches, nesting areas, and various flooring material to allow for dust bathing and foraging (Mench, Sumner, & Rosen-Molina, 2011). Both system designs, non-caged and enriched, increase the number of hens contained in one area, dramatically increasing contact between conspecifics. Non-cage systems house thousands to tens of thousands of hens and standard commercial enriched caged systems house 20 to 60 (Mench, Sumner, & Rosen-Molina, 2011). While these

newly developed housing systems allow hen's greater access to natural behaviors they also create new animal welfare concerns and economic challenges.

**Feather Pecking**

The primary animal welfare concern that has increased within these new housing systems is feather pecking (FP). Feather pecking is a welfare issue in all commercial housing systems, however this problem is magnified in systems where hens are housed in large numbers, giving pecking birds' greater access to conspecifics (Keeling, 1995, as cited in Lambton, Knowles, Yorke, & Nicol, 2010). This behavior ranges from gentle feather pecking (GFP), where one hen pecks at a conspecific's plumage without removing feathers, to severe feather pecking (SFP), a more violent pecking behavior that results in feather removal (Brunberg, Jensen, Isaksson, & Keeling, 2011; Parmentier, Rodenburg, De Vries Reilingh, Beerda, & Kemp, 2009). GFP behavior can occur as early as the day after hatch (Riedstra & Groothuis, 2002), although it is not injurious to the recipients plumage, there is evidence to support that even early GFP may increase risk for more severe FP in the future (Nicol, et al., 2013). SFP tends to develop as the hens mature, increasing throughout the laying period (Nicol, Gregory, Knowles, Parkman, & Wilkins, 1999); it is painful to recipients and results in stress, feather loss and wounded birds (Brunberg, Jensen, Isaksson, & Keeling, 2011; Heerkens, et al., 2015). In extreme cases, the physical injuries inflicted by this deleterious behavior may lead to cannibalism (Daigle, Rodenburg, Bolhuis, Swanson, & Siegford, 2014).

Injurious feather pecking (IFP) behaviors have a significant economic impact on the egg production industry. The reduction in plumage coverage caused by SFP reduces hen's thermal insulation, thereby reducing food conversion ratios (Nicol, et al., 2013). In order to maintain

their body temperature, bald chickens need up to 40% more feed (Blokhuis et al., 2007, as cited in Nicol, et al., 2013). Reduced efficiency at converting energy from feed into egg mass decreases egg production and diminishes profits (Lambton S. L., Knowles, Yorke, & Nicol, 2010; Nicol, et al., 2013). Hens receiving SFP, identified as 'pariah birds', are less likely to access food and water due to stress caused by repeated injurious pecks (Nicol, et al., 2013), further limiting them from reaching nutritional optimums. Cannibalism and increased flock mortality are also contributors to reduced profits as a consequence of elevated IFP behaviors (Lambton S. L., Knowles, Yorke, & Nicol, 2010). Concerns about hen welfare and economic efficiency have motivated egg producers to explore the causation of this behavior in an effort to discover potential methods for reducing it in non-cage housing systems.

Currently, FP is managed in non-caged systems by use of beak modification (infra-red or hot blade techniques) and through the manipulation of lighting regiments (Lambton S. L., Knowles, Yorke, & Nicol, 2010; Dennis, Fahey, & Cheng, 2009). In adult birds beak trimming is thought to reduce SFP thereby improving plumage condition (Staack et al., 2007, as cited in Nicol, et al., 2013). However, it has been observed that even in beak modified flocks, FP and plumage damage is still evident at high levels (Lambton, et al., 2013). In some cases it has been reported that plumage damage actually increased in pullets receiving beak modification as opposed to pullets with intact beaks (Staack et al., 2007 as cited in Nicol, et al., 2013).

Because excessive light intensities have been identified as a contributing factor in increased FP behavior (Kjaer & Vestergaard, Development of Feather Pecking in Relation to Light Intensity, 1999) some commercial farms select for light of certain wavelengths (red, blue or ultraviolet) or reduce light intensities to address the problem of injurious pecking behaviors (Mohammed et al., 2010, as cited in Nicol, et al., 2013). These modifications in lighting are

employed with the intent of reducing the bird's perception of color and detail of their conspecifics plumage and to lower the overall activity level of the flock (Bright, 2007; Nicol, et al., 2013).

While these methods are extensively used throughout the egg production industry, there are animal welfare concerns associated with each. Beak modification is suspected of causing both acute and chronic pain to the hen (Kuenzel, 2007; Lambton S. L., Knowles, Yorke, & Nicol, 2010), as well as changes in beak sensitivity and function (Freire, Eastwood, & Joyce, 2011; Nicol, et al., 2013). There are also questions regarding the effectiveness of beak modification to control IFP (Nicol, et al., 2013). Beak trimming is considered a mutilation in the European Union (EU) (Council directive 199/74/EC) and many member states support banning the procedure for more animal friendly alternatives. Light manipulation produces unintended welfare challenges as well, specifically abnormal eye development and function (Prescott, Wathes, & Jarvis, 2003; Lambton S. L., Knowles, Yorke, & Nicol, 2010). The undesirable aspects associated with common methods used to control FP in open housed systems combined with a growing consumer market that is critical of current husbandry practices has encouraged research into the underlying motivation for injurious pecking behavior and potential alternatives in reducing its occurrence (Nicol, et al., 2013).

**Motivation of Feather Pecking Behavior**

The motivation behind FP is complex and contains many different elements. While the underlying causes are not fully established, there is evidence to suggest that FP is a redirected pecking behavior related to the bird's motivation to forage (Blokhuis H. , 1986; Huber-Eicher & Wechsler, 1998; Brunberg, Jensen, Isaksson, & Keeling, 2011). An inverse relationship between

foraging behavior and FP has been described with an emphasis on time spent foraging and quality of foraging material (Zeltner, Klein, & Huber-Eicher, 2000). A framework of contributing factors includes: social, nutritional, physical, genetic and immunological (Parmentier, Rodenburg, De Vries Reilingh, Beerda, & Kemp, 2009). FP is the physical manifestation of influences from each of these factors acting on the bird in varying degrees with varying impact. This makes the task of reducing this unwanted behavior quite challenging.

It has been shown that individual chick behavior can be influenced by observing conspecifics (Johnston, 1998). Because FP occurs in groups directed at others in the flock, some FP behavior could be attributed to a learned behavior acquired by observing conspecifics who engage in FP (Zeltner, Klein, & Huber-Eicher, 2000). This implies that FP develops in one or a few birds within the flock and is then socially transmitted throughout the group (Zeltner, Klein, & Huber-Eicher, 2000). This social element underscores the importance of identifying the factors that contribute to FP behavior within individual birds.

Diet and nutrition have been found to impact FP frequency. Laying hens will adjust their eating times according to the energy level of the feed provided; low energy density feed expands feeding times and lowers the incidence of FP (Brunberg, Jensen, Isaksson, & Keeling, 2011; van Krimpen, Kwakkel, van der Peet-Schwering, den Hartog, & Verstegen, 2008). Feed high in protein and amino acid levels have a positive effect on plumage and pecking behavior (Ambrosen & Petersen, 1997), whereas feed low in mineral content is suspected of elevating the FP problem (Hughes & Duncan, 1972). Dietary tryptophan supplementation has been indicated to reduce FP (van Hierden, de Boer, Koolhaas, & Korte, 2004a). Tryptophan is a precursor to the monoamine neurotransmitter serotonin (5-hydroxytryptamine: 5-HT) and low 5-HT neurotransmission is associated with high FP behavior (van Hierden, Koolhaas, & Korte, 2004b).

Another dietary element associated with FP is feather eating. Feather eating can increase the rate of feed passage and may be a dietary effect associated with FP (Harlander-Matauschek, Piepho, & Bessei, 2006).

Evidence suggests that pecking preferences are established early in life (Sanotra, Vestergaard, Agger, & Lawson, 1995) and that exposure to suitable foraging material during rearing can reduce FP (Blokhuis & van der Haar, 1989; Green, Lewis, Kimpton, & Nicol, 2000). Studies identified a myriad of environmental influences that contribute to the rate of FP including: limited outdoor access with exposure to direct sunlight, number of personnel inspecting flock, temperature and type of drinking apparatus just to name a few (Green, Lewis, Kimpton, & Nicol, 2000; Nicol, Pötzsch, Lewis, & Green, 2003). It needs to be noted that exposure to these factors does not universally influence hens the same way. As previously mentioned FP initially appears in a restricted number of individuals and then is socially transmitted throughout the flock. With this in mind, it is beneficial to explore physiological mechanisms that may predispose layers to FP behavior at an individual level.

Different breeds of layers perform FP at varying rates, suggesting a genetic component to the behavior (Kjaer & Sørensen, 1997; Brumberg, Jensen, Isaksson, & Keeling, 2011). Buitenhuis, et al. (2003) performed a quantitative trait loci (QTL) study, which identifies polymorphisms in the DNA and associates them with a behavior, and found a QTL associated with FP behavior on chicken chromosome 2 (GGA2). A whole-brain gene expression study also analyzed the transcriptome of a high FP selection line compared to a moderate FP line and found 456 genes to be differentially expressed (Labouriau, Kjaer, Abreu, Hedegaard, & Buitenhuis, 2009). Brunberg, Jensen, Isaksson, & Keeling (2011) utilized chicken genome microarrays in order to evaluate hypothalamic gene expression and was able to identify 11 differentially

expressed transcripts between FP instigators and victims. The genes associated with these transcripts, specifically *TNFSF15*, *LAG3*, *ABCB1* and *MAPK8*, have been identified as having both direct and indirect roles in immune function, intestinal nutrient absorption, and glucose homeostasis (Brunberg, Jensen, Isaksson, & Keeling, 2011).

These findings support previous studies that suggest a link between FP and the immune system (Biscarini, et al., 2010; Buitenhuis, et al., 2004; Hughes & Buitenhuis, 2010; Parmentier, Rodenburg, De Vries Reilingh, Beerda, & Kemp, 2009). Buitenhuis et al (2004) found that there was a high genetic correlation between severe FP and primary antibody response to keyhole limpet hemocyanin (KLH) elucidating the idea that a selected line for high FP White Leghorns showed greater levels of specific humoral immunity than that of a low FP line. This connection however does not seem to be direct as Parmentier et al (2009) did not find a "direct relationship between the degree in which the Ab-producing component of the immune system is activated and FP". This topic of immune response needs to be explored in greater detail in order to identify elements that may contribute to anxiety, stress and increased FP behavior.

**The Role of Immune Response in Feather Pecking Behavior**

As previously indicated, evidence suggests that the relationship between immune response and FP is not a direct one (Parmentier, Rodenburg, De Vries Reilingh, Beerda, & Kemp, 2009), this necessitates an investigation into indirect factors affiliated with an immune response that may play a role in FP behavior.

In mammals, activation of the innate immune system initiates the synthesis and release of proinflammatory cytokines (Parmentier, Rodenburg, De Vries Reilingh, Beerda, & Kemp, 2009) which bind to specific receptors located on target cells and, through signal-transduction

pathways, alter gene expression (Goldsby, Kindt, Osborne, & Kuby, 2003). These low-molecular weight proteins are secreted by various cell types in response to certain stimuli with the purpose of mediating cell to cell interactions that are necessary to develop an effective immune response (Goldsby, Kindt, Osborne, & Kuby, 2003). Cytokines signal the brain of an urgent immune reaction through humoral and neural mechanisms which elicit a behavioral response known as "sickness behavior" (Berghman, 2016). Many studies have suggested a relationship between cytokines and reduced or altered cognitive function such as abnormal immune activation response and schizophrenia in humans (Wilson, et al., 2018).

Interferon-γ (INF-γ) is an example of a proinflammatory cytokine that is suspected of affecting cognitive ability through its role in the kynurenine pathway. The kynurenine pathway catabolizes tryptophan in order to synthesize nicotinamide adenine dinucleotide (NAD) (Davis & Liu, 2015). INF-γ is thought to be the predominant cytokine responsible for activating indoleamine 2,3-dioxygenase (IDO), an enzyme which converts tryptophan to kynurenine in the rate-limiting first step of the pathway (Wilson, et al., 2018). Because INF-γ induces IDO, an increase in immune function can escalate IDO activity thereby producing more kynurenine neuroactive metabolites that have the ability to cross the blood brain barrier such as 3-hydroxykynurenine (3-HK), Kynurenic acid (KYNA), quinolinic acid (QUIN), and picolinic acid (PIC) as illustrated in Figure 1 (Wilson, Demyanovich, Rubin, Wehring, Kilday, & Kelly, 2018; Lahiri, Dhaware, Singh, Panchagnula, & Ghosh, 2019). While KYNA and PIC are considered neuroprotective, 3-HK and QUIN function as neuronal excitotoxins and have been associated with neurodegenerative and neuropsychiatric disease in humans (Lahiri, Dhaware, Singh, Panchagnula, & Ghosh, 2019). Highly reactive free radicals are generated by 3-HK that lead to excitotoxic injury of the neuron and QUIN is a potent antagonist of N-methyl-D-aspartate

(NMDA) receptors that can induce glutamatergic hypo-functioning and contribute to cognitive dysfunction (Lahiri, Dhaware, Singh, Panchagnula, & Ghosh, 2019). KYNA, while considered neuroprotective, can also negatively affect cognition if produced in abundance. Similar to QUIN, KYNA acts as an endogenous antagonist of NMDA glutamate receptors, and in excess, can contribute to the negative effects of hypoglutamatergic disorders such as schizophrenia (Wilson, et al., 2018).

Through the activation of IDO, INF-γ has also been identified as a factor in the disruption of the serotonergic system. This is important because the serotonergic system contains central 5-HT, a neurotransmitter and peripheral signaling molecule that has significant influence over aggressive and impulsive behavior with, "diffuse afferent projections from the raphe nuclei to various brain areas" (de Haas & van der Eijk, 2018; Dennis & Cheng, 2014). This is relevant to INF-γ because 5-HT shares the amino acid tryptophan as a precursor with the kynurenine pathway (Parmentier, Rodenburg, De Vries Reilingh, Beerda, & Kemp, 2009). If INF-γ increases IDO activity, elevating catabolism of dietary tryptophan, serum levels can become diminished limiting its availability to be utilized in the synthesis of both peripheral and central 5-HT and potentially decreasing central 5-HT neurotransmission (Parmentier, Rodenburg, De Vries Reilingh, Beerda, & Kemp, 2009). This is significant with regards to behavior because central 5-HT plays a role in mood and dopamine activity (3,4-dihydroxyphenethylamine: DA) in various brain regions (de Haas & van der Eijk, 2018). Dysfunction of central 5-HT and DA activity has been shown to be a key factor in many mental disorders (Yildirim & Derksen, 2013). Low activity of central 5-HT is associated with excessive aggression, as well as, impulsive and compulsive behavior (de Haas & van der Eijk, 2018).

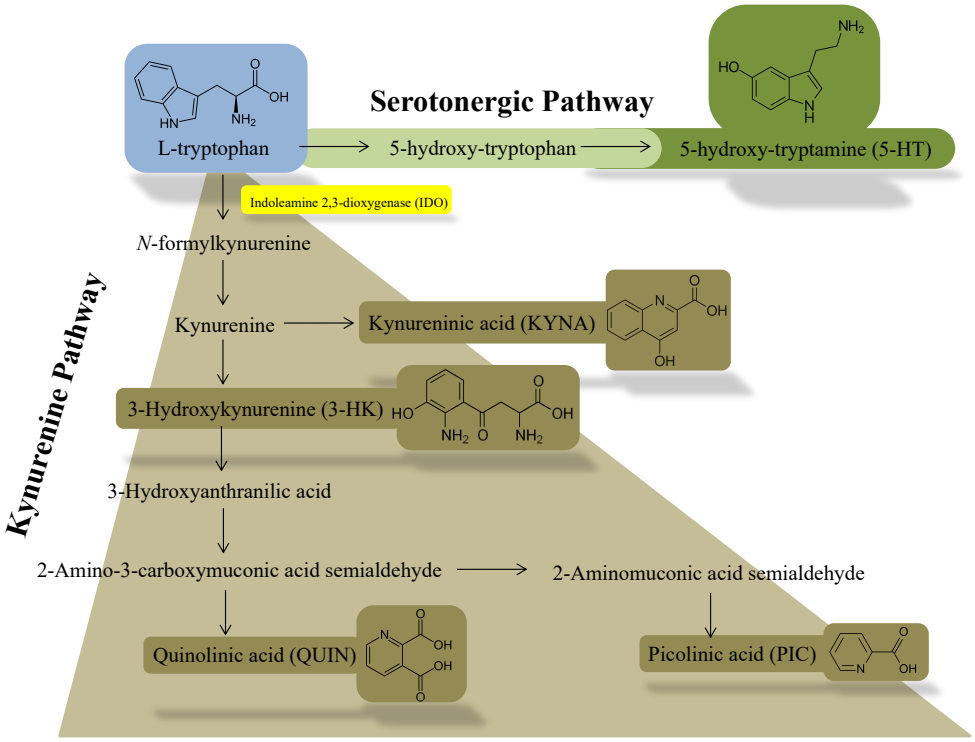



**Figure 1.** Pathways that utilize L-tryptophan. The Serotonergic pathway utilize L-tryptophan to produce 5-HT (serotonin). The kynurenine pathway utilizes L-tryptophan to produce nicotinamide adenine dinucleotide.

Proinflammatory cytokines also affect FP behavior by activating the hypothalamic-pituitary-adrenal (HPA) axis. In many species, Interleukin-1 (IL-1) has been identified as a potent HPA axis stimulator along with cytokines such as Interleukin-6 (IL-6), Interleukin-10 (IL-10) and tumor necrosis factor-α (TNF- α) (Dunn, 2007). While IL-6, IL-10 and TNF- α show the same activation capability with regards to the HPA axis, they act in a reduced capacity (Dunn, 2007). These cytokines can affect the activity of neurotransmitters like norepinephrine and corticotropin-releasing factor (CRF) in the central nervous system (CNS) modulating behavior (Dunn, 2007).

**The HPA Axis**

One of the major roles of the neuroendocrine system is to maintain homeostatsis in the presence of adverse stimuli (stress). This is executed through the activation of an intricate range of responses that include interactions between the endocrine, nervous and immune systems (Smith & Vale, 2006). Within this system, homeostasis is maintained through an allostasis response that includes fluctuations in glucocorticoids, cathecolamines, cytokines, behavior, heart rate, blood pressure, and antibody titers (Blas, 2015). Collectively, the HPA axis and the sympathetic branch (SNS) of the autonomic nervous system work together to modulate responses to external and internal stimuli that threaten to disturb the body's homeostasis (de Weerth, 2017). These processes are referred to as the stress response and have behavioral and physiological effects. The principle physiological stress system and associated anatomic structures that mediate this response are referred to as the HPA axis. The cellular components of the HPA axis are localized in the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland and the adrenal gland (Spencer & Deak, 2017). These cells synthesize hormonal signals that facilitate functional interactions of the system and ultimately produce effector glucocorticoid hormones (Smith & Vale, 2006; Spencer & Deak, 2017). In *Gallus gallus domesticus* the primary glucocorticoid hormone is corticosterone (CORT). CORT is a considerably influential regulator of many physiological systems and is utilized by virtually every cell in the body (Spencer & Deak, 2017).

The HPA axis is a three-tiered system comprising the hypothalamus, pituitary and adrenal gland. When stress is perceived hypophysiotropic neurons located in the medial parvocellular subdivision of the PVN synthesize and release corticotropin-releasing factor (CRF)

(Smith & Vale, 2006). CRF is the primary regulator of the HPA axis and when released travels into hypophysial portal vessels that lead to the anterior pituitary gland. CRF then binds to CRF type 1 receptors on endocrine cells of the anterior pituitary called corticotropes initiating cyclic adenosine monophosphate (cAMP) pathway events that release adrenocorticotropic hormone (ACTH) into circulation (Smith & Vale, 2006). Systemic migration of ACTH to melanocortin type 2 receptors (MC2-R) located in parenchymal cells of the adrenal cortex stimulates steroidogenesis and secretion of glucocorticoids located in the zona fasciculate (Smith & Vale, 2006).

Glucocorticoids and the HPA axis are both components of a system responsible for affecting changes in biological systems through the regulation of energy flow (Carsia, 2015). Noxious stimuli elicit a stress response that increases glucocorticoid secretion. This inhibits physiological processes such as growth, feeding, digestion and immunity, while increasing intermediate metabolism, cardiovascular tone and respiratory rate (Smith & Vale, 2006). Elevating CORT levels in a biological system increases blood sugar by initiating gluconeogenesis, suppresses immune function by reducing cytokine activity and increases fat and protein metabolism (de Weerth, 2017). Products of the SNS and HPA axis, such as CORT, epinephrine and norepinephrine prepare the animal for a "fight or flight" response (de Weerth, 2017). Behavioral alterations associated with this "fight or flight" stress response include a reduced perception of pain, increased awareness, improved cognition and euphoria (Smith & Vale, 2006). The stress response also affects behavioral processes that are not essential to immediate survival such as migration and reproduction (Blas, 2015).

It is important to note that glucocorticoids such as CORT have other biological functions outside of the stress response. They are fundamental to many body processes and regulation of

energy balance, physiology, morphology and behaviors that are not associated with the stress response (Blas, 2015). Three distinct temporal patterns describe HPA axis activity and subsequent CORT secretion: basal ultradian pulse, basal circadian fluctuation and stimulus induced activity (stress response) (Spencer & Deak, 2017). During basal ultradian pulse activity the HPA axis releases CORT in a very prominent diurnal rhythm (approximately every 60 min); these levels fluctuate in daily and seasonal patterns in order to easily optimize and maintain homeostasis within narrow limits (Blas, 2015; Spencer & Deak, 2017). CORT pulses are modulated by a number of factors that include circadian phase, sex, age and breed. In basal circadian fluctuation, CORT acts as a key mediator of circadian regulation of physiological function with its highest concentration levels associated with the onset of the circadian active period (Spencer & Deak, 2017).

The products of the HPA axis are released in a highly controlled manner and regulated through negative feedback loops that maintain hormone levels within narrow target physiological concentrations (de Weerth, 2017). This highly controlled release is due to the massive influence of glucocorticoids over energy regulating systems. Many elements of the neuronal and endocrine systems must work in coordination to regulate the magnitude and duration of HPA activity (Smith & Vale, 2006). This is important because activation of the HPA axis is intended to produce a short-term adaptive effect within the system. Prolonged activation of the HPA axis produces elevated CORT levels that, if present for an extended duration, could potentially contribute to the development of pathologies (Smith & Vale, 2006).

Dysregulation in the HPA axis is suspected of playing a role in human psychopathologies, such as autism, anxiety disorders, depression, and schizophrenia (de Weerth, 2017). This data along with evidence from several animal models has led to wide spread

assumptions that early life internal and external stressors influence changes in behavioral and emotional stress response that are carried into adulthood (de Weerth, 2017). This early life stress, or lack thereof, may alter the development of HPA axis functionality, affecting CORT levels affiliated with basal circadian fluctuation and stimulus induced activity creating a stress response that is disproportional to stimuli (de Weerth, 2017).

One of the earliest and most influential factors that may affect HPA programming is the establishment of early gut microflora. During the first years of life the gut microbiota and HPA axis develop and evolve rapidly. In this early developmental window bacterial colonization impacts gut physiology which increases the production of certain neurotransmitters such as 5-HT and γ-aminobutyric acid (GABA) as well as elevating expression of various cytokines (Heijtz, et al., 2011). These changes in gut physiology are integral to gut homeostasis and may assist in early programming of the HPA axis (Ghaisas, Maher, & Kanthasamy, 2016). "Developmental programming" is a term given to early life influences that impact an organism's development, structure and function (Heijtz, et al., 2011). Examples of development programming that have an impact on HPA function include animals who have been exposed to early handling by humans exhibiting dampened HPA response to stress compared to non-handled animals (Meaney, Aitken, Bhatnagar, Van Berkel, & Sapolsky, 1988) and an increased HPA response in adult animals who have experienced prolonged early life maternal withdraw (Schmidt, Oitzl, Levine, & de Kloet, 2002).

The colonization and establishment of gut microflora occurs concurrently within a developmental window of sensitive HPA programming and its role in influencing stress reactivity can affect stress response for the life of the organism (Foster & Mcvey Neufeld, 2013). Research by Sudo, et al., 2004 showed that germ-free (GF) mice displayed an exaggerated

CORT and ACTH response to restraint stress as opposed to house-specific pathogen-free (SPF) mice giving validity to the idea that colonizing microbes assist in the development of an HPA response to restraint stress. This is important because it supports the idea of a bidirectional relationship between gut microbes and the brain. It was also shown through this study that GF mice had reduced levels of brain-derived neurotrophic factor (BDNF), which is a protein and growth factor that promotes synaptic growth and controls synaptic plasticity and transmission (de Weerth, 2017; Sudo, et al., 2004). Sudo, et al. also shed light on the idea that gut microbes make this impact early on in the development of the HPA stress response by partially correcting the exaggerated HPA stress response through reconstituting GF mice with feces from SPF mice (Sudo, et al., 2004). This reconstitution however was only effective when performed at an early developmental stage emphasizing the idea of commensal bacteria assisting in neurodevelopment.

**The Microbiome-Gut-Brain Axis**

The gut microbiota is made up of many microbes including archaea, fungi, protozoa and most abundantly, bacteria (de Weerth, 2017). The approximate number of bacteria inhabiting the gut of an adult human is a hundred trillion; this means that the roughly two pounds of bacteria that reside within our intestines is ten times greater than the number of cells that make up our entire body and the unique gene set affiliated with these microbes is at least one hundred fifty times larger than the human gene set (de Weerth, 2017; Qin, et al., 2010; Sudo, et al., 2004). This expanded gene repertoire provided by gut microbes includes many genes that encode functions that are complementary to the hosts genome, enhancing biological capabilities with numerous features that the host did not have to evolve itself (Walker, 2013). Beyond their influence on early HPA programming, commensal microbes play many integral roles in the postnatal development of their hosts. Most notably, they are involved in promoting host immune

development, nutrient processing and limiting pathogen colonization by occupying gut binding sites (Sudo, et al., 2004; Heijtz, et al., 2011; Ding, et al., 2017). These gut microbes also play a role in influencing the hosts brain function and behavior.

The extent of communication and its impact on neurological function as well as behavior between the gut microbiome and the brain remains complex and relatively unknown. There are however several known pathways for communication to occur between gut microbes and the brain during development including the parasympathetic nervous system, microbial endocrinology, immune system and direct delivery of gut microbial products such as neuroactive metabolites and neurotransmitters through the circulatory system (Alam, Abdolmaleky, & Zhou, 2017).

The principle element of interaction between the gut microbiome and the brain in regard to the parasympathetic nervous system is the vagus nerve (VN). The VN is the longest cranial nerve in the body with roles in controlling cardiovascular, respiratory, immune and endocrine systems. It also plays a major role in gut physiology and provides both excitatory and inhibitory control over gastric, intestinal and pancreatic functions (Browning, Verheijden, & Boeckxstaens, 2017). There is also evidence that the VN communicates with the immune system through the detection of inflammation resulting in changes in appetite, mood and sickness behavior eliciting an efferent vagal signal that ultimately plays a role in the modulation of the immune response (Browning, Verheijden, & Boeckxstaens, 2017). The VN provides parasympathetic innervation to the GI tract establishing a connection with the enteric nervous system (ENS) and there is growing evidence that gut microbes utilize the ENS to modulate gut-brain signaling (Browning, Verheijden, & Boeckxstaens, 2017). This innervation is not homologous along the length of gut and parallels the variances in microbial population found throughout (Lyte, 2013). This provides

a direct neurochemical pathway for microbial-brain interaction to occur from the GI tract (Sampson & Mazmanian, 2015). Activation of vagal afferents by gut microbes can be initiated through several mechanisms. Direct activation can be instigated in situations where gut permeability is compromised and indirect activation can occur through the stimulation of enteroendocrine cells or gut associated lymphoid tissue that produce neuroactive mediators (Browning, Verheijden, & Boeckxstaens, 2017). Evidence suggesting VNs role in gut-brain communication has been presented through a study showing elevated CORT plasma as well as c-Fos mRNA expression levels of the paraventricular nucleus (a measure of neuronal activity) in *Bifidobacterium infantis* inoculated GF mice (Sudo, et al., 2004). This is significant because these elevated levels were found to precede increased serum levels of IL-6 and were repeated in GF mice pretreated with anti-IL-6 antibody indicating a humoral cytokine-independent pathway (Sudo, et al., 2004). As further support of the VNs role in bidirectional signaling between gut microbes and the brain, mice who were administered *Citrobacter rodentium* showed a VN dependent increase in anxiety-like behavior and conversely mice with elevated anxiety-like behavior due to nematode-induced GI inflammation normalized (also in a VN dependant manner) after receiving *Bifidobacterium longum* NC3001 (Bravo, et al., 2011; Bercik, et al., 2011).

Another route of gut-brain interaction can be found in microbial endocrinology. This mode of communication is facilitated though commensal bacteria's ability to synthesize and recognize neurochemicals that are exactly analogous in structure to those of the hosts neurophysiological system (Lyte, 2013). This bidirectional crosstalk between microbes and hormones can affect host metabolism, immunity and behavior. (Neuman, Debelius, Knight, & Koren, 2015). Many neuroendocrine hormones identical in structure and biochemical synthesis

pathways are found in nature and it has been proposed that the existence of neurochemical-based cell-to-cell signaling pathways in vertebrates is due to late horizontal gene transfer from bacteria (Lyte, 2013). Clues that this could be a pathway of gut-brain communication came from the observance that bacteria perform quorum sensing as a mode of communication which utilizes the synthesis and recognition of autoinducer molecules (Neuman, Debelius, Knight, & Koren, 2015). These hormone-like autoinducer molecules are used by bacteria in order to regulate functions such as motility, virulence and coordinate bacterial growth (Neuman, Debelius, Knight, & Koren, 2015). Some of the molecules also have the potential to modulate host cell signal transduction through the host's hormones, activating signaling pathways (Karavolos, Winzer, Williams, & Khan, 2013). Two types of hormones have been proposed for candidates in gut microbiome-brain interaction, neurohormones such as 5-HT, dopamine, epinephrine and norepinephrine and stress hormones which include CORT, ACTH and corticotropin (Neuman, Debelius, Knight, & Koren, 2015). Gut microbes possess the capability to both produce (directly or indirectly) and respond to 5-HT, dopamine and norepinephrine (Roshchina, 2010). Low 5-HT neurotransmission, as discussed earlier in the Motivation of Feather Pecking Behavior section of this review has been associated with high feather pecking behavior. This is significant because 90% of the hosts 5-HT in mammals is found in the intestines and can be influenced through diet (Neuman, Debelius, Knight, & Koren, 2015). It also showcases the expansive physiological and neurological influences of 5-HT, with roles in regulating intestinal movement, appetite, sleep, mood and behavior (Neuman, Debelius, Knight, & Koren, 2015; de Haas & van der Eijk, 2018). This lends support to the concept that microbial endocrinology is a potential influencer of mood and behavior.

As discussed earlier, the immune system has an indirect connection to feather pecking behavior. This has relevance because the host's immune response is shaped and modulated by the gut microbiome. In chickens, early gut colonization of commensal microbes initiates an innate immune reaction that is represented by increased expression of IL-8 and IL-17in the first week of life (Crhanova, et al., 2011). This initiates infiltration of heterophils and lymphocytes into the lamina propria or gut epithelium and ultimately to the normalization of the gut immune system (Crhanova, et al., 2011). Host immune cells recognize microbial molecular motifs, such as bacterial lipoprotein (BLP) or lipopolysaccharide (LPS) by way of pattern recognition receptors (PPRs) (Alkie, Yitbarek, Hodgins, & Kulkarni, 2019). When Toll-like receptors (TLRs), which are a class of PRRs, encounter pathogen associated molecular patterns (PAMPs) it activates signaling pathways found in innate immune cells such as macrophages, heterophils and dendritic cells (Alkie, Yitbarek, Hodgins, & Kulkarni, 2019; Sampson & Mazmanian, 2015). This leads to the synthesis of chemokines, cytokines and host defense peptides (HDPs) which facilitate specific cell and antibody mediated immune responses (Alkie, Yitbarek, Hodgins, & Kulkarni, 2019).

The innate immune system represents an early mechanism for host defense against pathogenic infection. The primary components include physical barriers (gut mucosa), physiological mechanisms (fever), phagocytic cells and molecules of the complement system (antimicrobial peptides) (Berghman, 2016). This limited defense is necessary because the adaptive immune system is not yet developed at birth, taking up two weeks to take shape in a chicken (Berghman, 2016). While the innate immune response is effective, it is not as pathogen specific as the adaptive immune response causing unintended consequences to the host during development. As a result of innate immune activation numerous proinflammatory cytokines

such as IL-1 and IL-6 bind to receptors on the afferent branches of the VN promoting alterations in brain centers responsible for appetite, emotion, mood, and sleep (Berghman, 2016). Proinflammatory cytokines also migrate to the brain disseminating through the blood brain barrier (BBB) and influence local release of prostaglandins (Sampson & Mazmanian, 2015; Berghman, 2016). Further impact of these proinflammatory cytokines, with regard to feather pecking behavior and reduced or altered cognitive function, has been previously covered in The Role of Immune Response in Feather Pecking Behavior.

The gut microbiomes role in nutrient processing is well known, providing the host with beneficial carbohydrate, protein and fat utilization as well as vitamin synthesis. Normal brain development relies on metabolites produced from the developing gut microbiome in order to meet the metabolic demands during critical or sensitive periods of maturation. Gut microbes digest and ferment many nondigestible carbohydrates in the colon, producing short chain fatty acids (SCFAs) that are beneficial to host health. Butyrate, acetate, and propionate are examples of such SCFAs that have neuroactive properties, establishing another pathway of gut-brain interaction. These SCFAs migrate into the host's serum and have the capability of crossing the BBB (Sampson & Mazmanian, 2015). Acetate for example, once in the brain, alters the level of neurotransmitters such as glutamate, glutamine and GABA (Sampson & Mazmanian, 2015). This is relevant because GABA, as mentioned earlier, is suspected of playing a role in early HPA programming. Butyrate has a role in host energy metabolism and immune function and may modulate behavior (de Weerth, 2017). It has also been shown to assist in the repair of BBB integrity found in GF mice as a result of reduced expression of endothelial tight junction proteins (Sampson & Mazmanian, 2015). Furthermore, SCFAs can influence host chromatin structure and may play a role in levels of BDNF (de Weerth, 2017).

## Probiotics

In mammals, microbial gut colonization is suspected of starting in utero. Initially thought of as sterile, metagenomic studies have revealed that microbes are present in different regions of the placenta and that these microbes are suspected of being vertically transmitted from mother to infant (Ding, et al., 2017). In *Gallus gallus domesticus* the detection of microbes is routinely found in developing embryos (Ding, et al., 2017). While the details of early establishment and inheritance of the gut microbiome are unknown, early microbial exposure is suspected to occur during the formation of the fertilized egg in the oviduct (Ding, et al., 2017). The oviduct joins the urinary and digestive tracks in the cloaca making it a strong candidate for early microbial exposure (Ding, et al., 2017). It should be noted that the presence of these early microbes in both mammalian and avian systems is still being debated and while these microbes may be present during a critical developmental period, their impact on the developing fetus, diversity and extent of gut colonization is unknown. Further investigation needs to be performed in order to identify these microbes associated with the reproductive system and their potential impact on the developing organism. Beyond this limited, in utero, suspected microbial contact, construction of the gut microbiome is largely dependent on exposure to microbes present in the early postnatal environment as well as the birth canal, maternal skin flora and early feeding interactions (Ding, et al., 2017).

It is widely suspected that an early "healthy" microbiota resembles one that is naturally conveyed from the microbiota of healthy adult animals (Marcolla, Alvarado, & Willing, 2019). This creates a unique challenge for chickens raised in a commercial setting because they are hatched in "clean environments" with no exposure to potentially beneficial microflora from adult birds (Crhanova, et al., 2011). These "clean environments" are of strategic design, with a focus

on limiting the prevalence of *Salmonella enterica* in poultry. This leads to early microbial colonization with microbes of coincidence that may not offer all of the assistance required for healthy development (Crhanova, et al., 2011). It also increases the opportunity for early infiltration and prolonged proliferation of pathogenic bacteria into the gut (Crhanova, et al., 2011). As discussed throughout this review, early microbial colonization is paramount to host development and has a profound impact on the entire lifespan of an organism. Many of the topics previously covered illustrate the potential pathways and mechanisms early gut microbes utilize in order to assist in shaping host behavior and physiological response to environmental input. This underlines the importance of an early interaction with "healthy" bacteria to ensure critical microbes colonize the gut and establish a complex and stable community.

The importance of early gut colonization is not lost on the livestock industry. It is recognized that the gastrointestinal health of livestock animals is directly tied to production efficiency and several methods have been developed over the years to promote it (Marcolla, Alvarado, & Willing, 2019). One of the first methods was demonstrated by Nurmi and Rantala in 1973, in which *Salmonella infantis* infection was successfully reduced in broilers through the administration of mature chicken cecal contents to one day old chicks via oral gavage (Nurmi & Rantala, 1973). This showcased the importance of microbial colonization and contributed to the idea that animals raised in sanitized environments may lack commensal microbes that modulate inflammatory responses that lead to host survival and development (Marcolla, Alvarado, & Willing, 2019). Subsequently, this lead to the development of probiotic cultures devised specifically for supplying these beneficial microbes. Competitive exclusion cultures are just such a probiotic culture, administered early to post-natal or just hatched animals with the intent to reduce pathogenic infection through early colonization of the gut. This method of pathogenic

protection relies on the complexity and stabilization of gut commensal microbes to out compete potential pathogens for attachment sites and resources as well as supplying antimicrobial compounds and enhancing the host's immune system (Callaway, et al., 2008).

Beyond the prevention of pathogens, early supplementation of beneficial probiotics may also contribute to the reduction of aggressive pecking behavior and anxiety in chickens through the microbiome-gut-brain axis. Hsiao, et al. demonstrated that behavior abnormalities found in maternal immune activation (MIA) mice offspring were corrected through the use of *Bacteroides fragilis*. *B. fragilis* was shown to correct gut permeability, alter gut microbial composition and improve defects in communicative, anxiety-like and sensorimotor behaviors (Hsiao, et al., 2013) Interestingly, it was also demonstrated that these alterations occurred in the absence of persistent colonization of *B. fragilis*, suggesting that probiotics may influence abundance of specific groups of already established commensal bacteria who may relay beneficial functionality to the host (Hsiao, et al., 2013). This was also noted by McNulty et al. (2011), who found that probiotic supplementation with fermented dairy products did not alter the composition of the gut microbiome but rather altered the transcriptional state and/or metabolic activity of the microbiota (McNulty, et al., 2011).

**Electron Beam Technology**

In order to further explore the mechanisms of non-colonizing probiotic influence on an established commensal gut microbiota it is advantageous to develop a probiotic culture that is metabolically active yet unable to colonize. For this application electron beam (eBeam) processing is an ideal technology. EBeam is a form of ionizing radiation that prevents bacterial multiplication through direct and indirect DNA damage. Currently its primary application is in the food industry providing food pasteurization, pathogen elimination and phytosanitary

treatment, (Pillai, 2016) however its potential impact in other applications is far reaching and ever expanding. As such, eBeam is emerging as a critical technology in areas such as vaccine development, municipal sludge processing and biological research (Kogut, et al., 2012; Bhatia & Pillai, 2019).

EBeam processing allows for ionizing radiation to be applied to target material in a controlled manner. Unlike other forms of ionizing radiation such as gamma radiation, eBeam is an on/off technology generating radiation from a linear or Rhodotron type accelerator rather than radioactive materials such as cobalt-60 and cesium-137 (Pillai, 2016). The high energy produced from these accelerators is capable of removing electrons from atoms (ionizing radiation) and produces a stream of electrons that when projected onto materials further jettisons electrons from atoms causing ionization events, which subsequently eject even more electrons from adjacent atoms (Pillai, 2016). Short lived yet highly reactive free radicals such as hydroxyl radicals, hydrogen peroxide, hydrogen, hydrated electrons and hydrated protons are also produced when water molecules are irradiated using ionizing radiation (Pillai, 2016). Together, the primary and jettisoned electrons along with the free radicals inflict numerous single- and double- stand breaks in the DNA of any microbes present (Pillai, 2016). The molecular damage renders the bacteria inactivated and unable to propagate. This damage however is apportioned to the DNA, as metabolic function and components of the bacterial cell membranes are preserved (Jesudhasan, et al., 2015; Bhatia & Pillai, 2019). This is a significant property of eBeam bacterial processing because it produces metabolically active yet non-culturable bacteria that can potentially be utilized in many biological applications.

Energy translates to the penetrating power of ionizing radiation and dose rate is the rate at which that energy is deposited to target material. The dose rate of a target material can be

manipulated though the time it is exposed to the electron beam (Pillai, 2016). D-10 values are a measure of specific bacterial resistance to ionizing radiation and provide information on the dose required to achieve a 90% reduction in the number of viable microbial cells (Pillai, 2016). The inactivation of defined titers of bacteria can be achieved using calculated delivered doses from defined D-10 results.

# CHAPTER III

# MATERIALS AND METHODS

## Animals and housing

Three identical pens (1.83 x 1.52 x 2.3 m) as shown in Figure 2, were constructed in six individual rooms at the USDA-ARS (USDA-ARS-FFSRU, College Station, TX, USA) totaling eighteen individual pens. Each pen was furnished with a hanging poultry feeder, perch (1.37 m) and water line containing six nipples. The floor was concrete covered in 3-6 inches of pine shaving litter and observed daily for wetness. Any caking or wet litter was removed as soon as noticed and pens were top-dressed with fresh shavings every 2-3 weeks. Temperature was regulated by central air conditioning and heat lamps (one 250 watt centrally located lamp per pen) and illumination was supplied through incandescent lights. Temperature, light intensity and light duration were adjusted according to commercial layer recommendations (Hy-Line, 2016) through-out hen maturation.

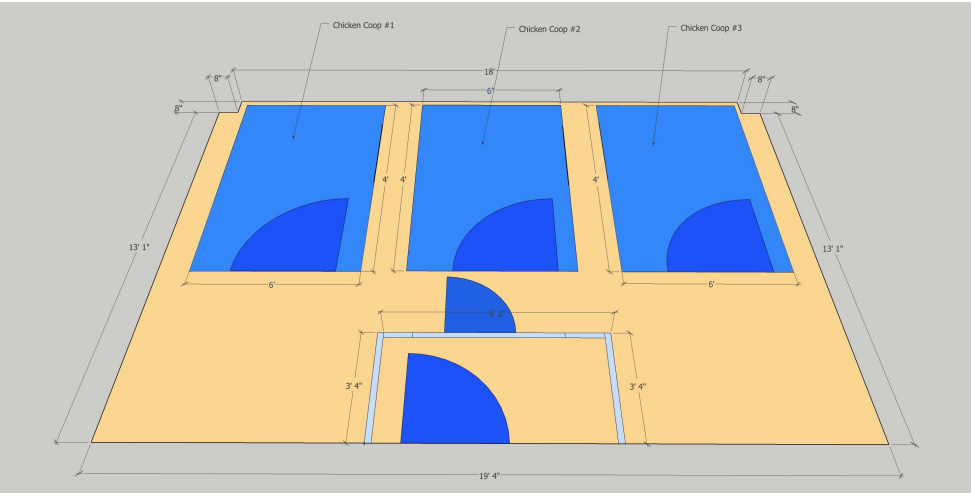



**Figure 2.** Diagram illustrating placement of pens within an individual room. Three pens were constructed in each room and each room contained one pen for each treatment group (culture, eBeam and water).

Two hundred and sixteen W-36 commercial layers sourced at day of hatch from Hy-Line International (Hy-Line North America, LLC, Bryan, TX, USA) were evenly distributed throughout the 18 pens totaling 12 chicks per pen. The chicks were feed pullet starter/grower (Producers, College Station, TX, USA) up to week 25 at which point they were transferred to lay crumble 17 (Producers, College Station, TX, USA) for the remainder of the experiment. At week 21 the hens were marked using spray paint in order to individually identify each bird in video recordings, sampling and behavior testing. All chickens were cared for in accordance with the United States Department of Agriculture Agricultural Research Service Institutional Animal Care and Use Committee guidelines.

**Continuous-flow culture**

A continuous flow (CF) culture was started on 02/18/2016 from a previously established (07/11/2012) recombined porcine-derived continuous flow (RPCF) culture developed from a CF porcine-derived competitive exclusion culture (PCF-1, Nisbet et al., 1999 U.S. Patent 5,951,977) at the USDA-ARS (USDA-ARS-FFSRU, College Station, TX, USA). RPCF was constructed by isolating, identifying and recombining the bacteria included in PCF-1, which was propagated from the cecal contents of a 6-week-old healthy pig (Harvey, et al., 2002). Nisbet et al. demonstrated that CE cultures rapidly colonize the gut of newly hatched chicks resulting in a 100 fold increase in microbial populations of 3 day old chicks as compared to untreated controls (Nisbet, et al., 1994). The RPCF culture was considered an appropriate choice for use in this experimental because both adult pigs and chickens are hindgut fermenters who receive similar diets in a commercial setting contributing to a similar cecal environment in terms of pH, oxidation reduction potential, anaerobicity, and bacterial population (Genovese, et al., 2003).

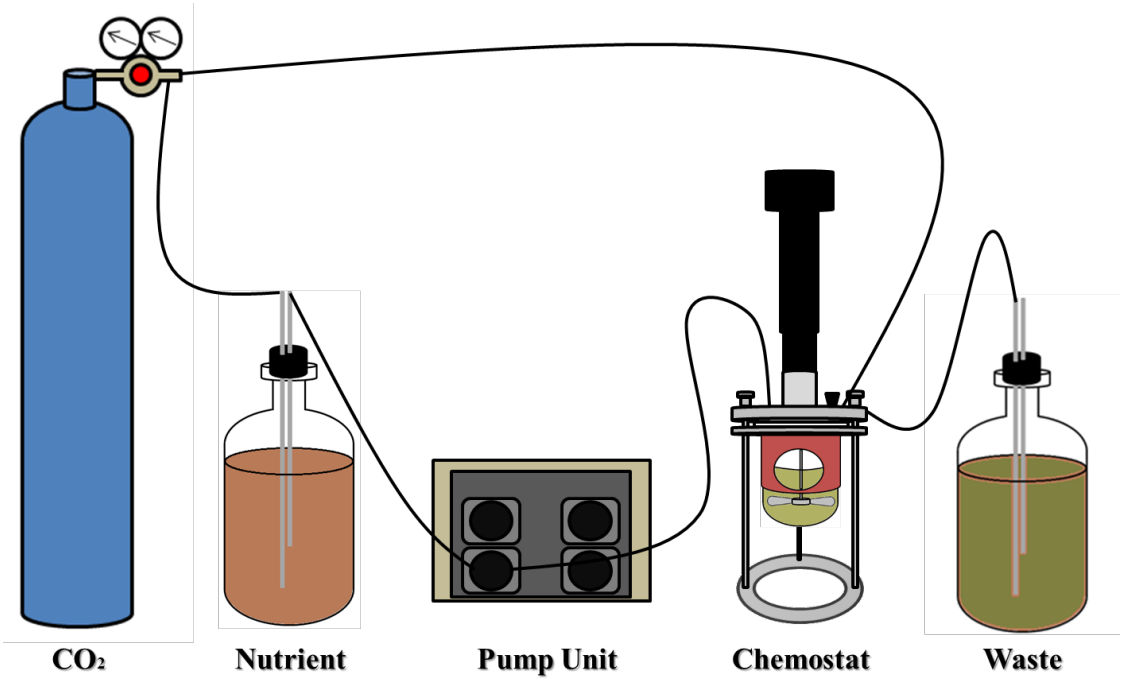



**Figure 3.** Diagram showing the components of a chemostat system. $CO_2$ gas is supplied to the nutrient media and chemostat. Nutrient is delivered to the chemostat in a controlled manner using a computerized pump unit. Waste is disposed of through the same pump unit.

The CF culture was maintained in a BioFlo 110 modular benchtop fermenter (New Brunswick Scientific Co, Edison, NJ, USA) fitted with a 2 L chemostat vessel as illustrated in Figures 3 and 4. Briefly, 500 ml of effluent collected from RPCF 07/11/2012 was delivered into the chemostat vessel and autoclaved (30 min, 121°C, 22 P). The vessel was then maintained in anaerobic conditions constantly being flushed with $O_2$-free $CO_2$ at a constant temperature of 39°C. After 24 hours, 50 mL of 07/11/202 RPCF was collected in a $CO_2$ purged conical tube and inoculated into the prepared chemostat vessel. The nutrient pump was turned on at a dilution rate of $0.0416^{-1}$ per hour (corresponding to a flow rate of 0.80 ml/minute and a vessel turnover time of 24 hr) and pH was monitored using an Orion 2 Star benchtop pH meter (Thermo Fisher,

Waltham, MA, USA). The nutrient supplied to the CF culture was Viande Levure (VL) broth medium, (tryptose [10 g/liter], beef extract [2.4 g/liter], yeast extract [5 g/liter], dextrose [2.5 g/liter], l-cysteine HCL [0.6 g/liter] and NaCl [5 g/liter]) it was prepared in 13-liter Pyrex bottles, autoclaved (1.5 hr, 121°C, 22 P) and flushed with a constant stream of $O_2$ free $CO_2$ immediately upon removal form the autoclave.

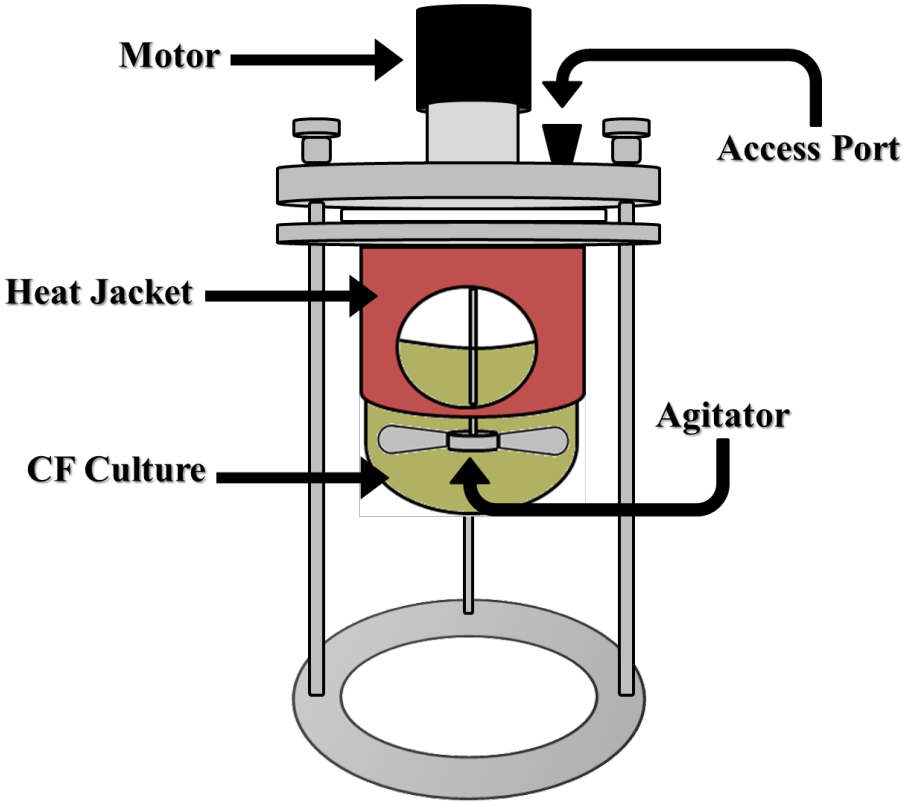



**Figure 4.** Illustration of chemostat unit housing constant flow culture.

**CF culture microbial diversity**

CF culture collected directly from the chemostat using a 50 ml conical tube flushed with $CO_2$ was transferred to a Bactron IV anaerobic hood (Sheldon Manufacturing, Cornelius, OR, USA) and a 5 ml aliquot was packaged for microbial diversity analysis using 16S rRNA Diversity Assay bTEFAP® illumina (MR DNA, Shallowater, TX, United States). Briefly, 16S rRNA gene V4 variable region PCR primers 515/806 with barcode on the forward primer were used in a 28 cycle PCR (5 cycles used on PCR products) reaction using HotStarTaq Plus Master Mix Kit (Qiagen, USA) with the following cycling program: 94°C for 3 minutes, followed by 28 cycles at 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Amplicon products were then used to prepare Illumina DNA library and sequencing was performed on a MiSeq (Illumina, San Diego, CA, USA) following manufacturer's guidelines. Sequence data was processed using MR DNA analysis pipeline, where data was cleaned and operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu).

**Electron beam (eBeam) irradiation**

The CF culture was collected in a 2 L Erlenmeyer flask flushed with $CO_2$ and directly transferred to an anaerobic hood for packaging. One hundred microliter aliquots were placed in Whirl-Pak® bags (Nasco, New York, NY, USA) and heat sealed. Each Whirl-Pak® bag was then triple bagged to meet University regulations regarding handling potentially biohazardous

samples within the eBeam irradiation facility. EBeam processing was performed at the National Center for Electron Beam Research located at Texas A&M University (College Station, TX, USA). Samples were irradiated using a LINAC style eBeam accelerator 10 MeV (energy), 18 kW (power). Alanine dosimeters (calibrated to international standards) were used to measure the delivered eBeam dose and were read using a Bruker E-scan spectrometer (Bruker, Billerica, Mass, USA). Samples had a target eBeam dose of 10 kGy and were evaluated for growth each week during supplementation (Table 1).

| Description of EBEAM Culture at Time of Supplementation | | | | | |
|---|---|---|---|---|---|
| Week | Date Delivered | pH | Anaerobic/Aerobic plate | Ebeam Session | Delivered Dose |
| 1 | 12/21/17 | 6.33 | No Growth | 12/19/17 | 10.45 kGy |
| 2 | 12/28/17 | 6.88 | No Growth | 12/19/17 | 10.63 kGy |
| 3 | 1/4/18 | 6.42 | No Growth | 12/19/17 | 10.65 kGy |
| 4 | 1/11/18 | 6.45 | No Growth | 12/19/17 | 10.82 kGy |
| 5 | 1/19/18 | 6.34 | No Growth | 1/17/18 | 10.30 kGy |
| 6 | 1/25/18 | 6.42 | No Growth | 1/17/18 | 10.35 kGy |
| 7 | 2/1/18 | 6.46 | No Growth | 1/17/18 | 10.25 kGy |
| 8 | 2/8/18 | 6.39 | No Growth | 2/6/18 | 10.71 kGy |
| 9 | 2/15/18 | 6.37 | No Growth | 2/6/18 | 10.79 kGy |
| 10 | 2/22/18 | 6.55 | No Growth | 2/6/18 | 10.28 kGy |
| 11 | 3/1/18 | 6.35 | No Growth | 2/6/18 | 10.53 kGy |
| 12 | 3/8/18 | 6.1 | No Growth | 3/6/18 | 10.44 kGy |
| 13 | 3/15/18 | 6.13 | No Growth | 3/6/18 | 10.52 kGy |
| 14 | 3/23/18 | 6.17 | No Growth | 3/6/18 | 10.52 kGy |
| 15 | 3/29/18 | 6.19 | No Growth | 3/6/18 | 10.77 kGy |
| 16 | 4/5/18 | 6.22 | No Growth | 3/6/18 | 11.31 kGy |

**Table 1.** Description of eBeam culture schedule and delivered dose. Table includes age of birds (week), date delivered, pH of the packaged EB culture and the results of anaerobic/aerobic plating of the aliquoted samples. Also listed is the eBeam session date and delivered dose associated with each sample.

The selected target dose of ~10 kGy was determined after evaluating the eBeam treated culture for bacterial growth and cell membrane integrity at different applied dosages (0 kGy, 2 kGy, 4 kGy, 8 kGy and 10 kGy). Growth of the inactivated CF culture was evaluated aerobically and anaerobically through direct plating of the eBeam treated culture in triplicate on Brucella blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA) for anaerobic counts and TSA II

with 5% sheep blood agar plates (BD Biosciences, Franklin Lakes, NJ, USA) for aerobic counts. The plating was performed in an anaerobic environment for both anaerobic and aerobic counts. Anaerobic plates were incubated at 37°C inside of the anaerobic chamber and aerobic plates were removed from the anaerobic environment after plating and incubated at 37°C. Plate counts were performed after a 24 hour incubation period for both anaerobic and aerobic plates.

Cell membrane integrity was visualized using the LIVE/DEAD® *Bac*Light ™ Bacterial Viability Kit (Molecular Probes®, Eugene, OR, USA). Briefly, this kit identifies bacteria with an intact cell membrane as fluorescent green, whereas bacteria with compromised cell wall integrity exhibit significantly less green fluorescence and often fluoresce red. This fluorescence is due to a mixture of two nucleic acid stains; a green-fluorescent SYTO® 9 stain that can penetrate cells with either intact or damaged cell membranes and a red-fluorescent propidium iodide stain which can only penetrate cells with damaged membranes (Hieke & Pillai, 2018).

Metabolic function of the ebeamed culture was also evaluated at a received dose of ~10 kGy using the alamarBlue® assay (Invitrogen, Grand Island, NY, USA). This assay monitors metabolic cellular activity through an oxidation-reduction (REDOX) reaction that occurs within the cell. Resazurin, a non-fluorescent compound incorporated into the alamarBlue dye, migrates into the interior of the cell and is reduced into resorufin, which is highly fluorescent. A cell that is viable and healthy will reduce more resazurin to resorufin, which produces more fluorescence indicating greater metabolic function as compared to dead or compromised cells (Hieke & Pillai, 2018). After ebeam processing the samples were stored in anaerobic jars at 4°C until being prepared for delivery to chickens on day of gavage.

## Treatments

Each of the three pens contained in a single room as shown in Figure 5, were assigned a different treatment group. The location of the assigned treatment groups was different in each room so as not to place the same treatment group in the same configuration for each room. The three treatment groups were as follows. (1) CF culture (CF) – chickens that received a weekly supplementation of CF culture directly sourced from chemostat; delivered through oral gavage within 30 minutes of collection. (2) eBeam irradiated CF culture (EB) – chickens that received a weekly supplementation of irradiated CF culture that was anaerobically packaged and processed using the eBeam at the National Center for Electron Beam Research at Texas A&M University (College Station, TX, USA). This culture was anaerobically stored at 4ºC after processing and allowed to reach room temperature before being delivered to chickens through oral gavage. (3) Water (WR) – control chickens that received a weekly supplementation of water delivered through oral gavage. Distilled water was autoclaved (30 Min, 121°C, 22 P) the morning of delivery and allowed to cool to room temperature.

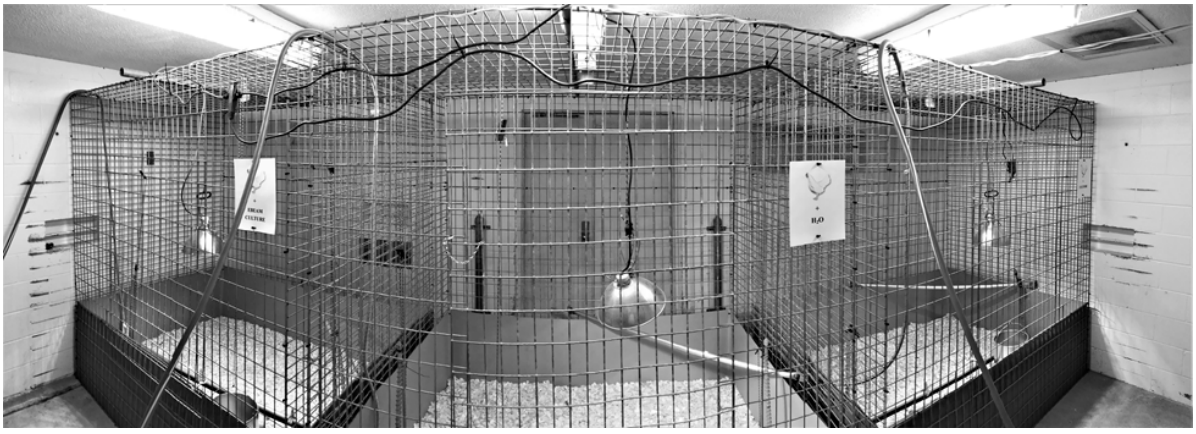


**Figure 5.** Image of three housing pens within a room. Each room contained one pen designated for each treatment group. The treatment groups were placed in different spatial arrangements from room to room. A gap between pens was incorporated into the design of the pens and the lower boarder of the pen was contained with brooder board.

The CF culture/eBeam Irradiated culture/Water supplementation was delivered to the chickens through oral gavage using a 10 ml syringe equipped with a 20 gauge dosing needle. Oral gavage was performed by the same trained personnel each week and was delivered to the birds for the first 16 weeks of the experiment starting at day of hatch. Briefly, the chicken was momentarily restrained in order to extend the chicken's neck and open its beak. The dosing needle was inserted behind the glottis towards the right side of the back of the mouth into the esophagus. Once positioned, the plunger of the syringe was slowly depressed delivering the supplement. After delivery the dosing needle was removed following the same path as insertion (Alworth & Kelly, 2014).

**Open-field test**

At week 4 of the experiment 32 pullets (n=12, CT; n=10, EB; n=10, WR) were randomly selected to undergo an open-field test as described by Rodenburg, et al. (Rodenburg, et al., 2004). The open-field test was performed by one person over 3 days between the hours of 07:20 and 23:20 in a testing room adjacent to rooms containing the birds home pens. Before testing, the floor of the testing arena and ambient room temperature were recorded. Each pullet was removed from their home pen and placed into a 20 x 12 x 20 cardboard transport box. The transport box was carried into a darkened testing room where the bird was removed and placed within the center square of a 25 square testing grid (each square measuring 20 x 20 cm) as shown in Figure 6. The grid was housed within a 1.2 x 1.2 m arena constructed of black foam PVC sheets and PVC pipe. Once the bird was placed in the center of the testing grid, the testing personnel exited the room and observed the test from a viewing station located in the hallway. A camera (4M AHD Analog Bullet Camera, SDC-89440BF, Hanwha Techwin, Seoul, South

Korea) mounted directly above the arena captured video and a digital video recorder (DVR)(SDR-B85300, Hanwha Techwin, Seoul, South Korea) located at the viewing station recorded the session. The test was initiated when the lights of the testing room were turned on. All birds were tested individually for 10 minutes. Focal sampling was used to record latency to first step, number of squares entered, number of steps taken and number of times the bird defecated during the session as described in Table 2.


**Figure 6.** Image of hen in open field testing arena

| Behavior | Description |
|---|---|
| Latency to first step | Measured time in seconds to hens first step during testing period |
| Number of squares entered | Number of times the hen traveled into a new square on the testing grid |
| Number of steps taken | Total number of steps taken by the hen during the testing period |
| Defecating | Number of times the hen defecated during the testing period |
| Preening | Number of times the hen engages in preening behavior (uses beak to rearrange, manipulate or clean body feathers) during testing period |

**Table 2.** Ethogram of open field test

The open-field test was repeated at week 35 using 72 chickens (n=24, CT; n=24, EB; n=24, WR) in a 25 square grid composed of 30.5 x 30.5 cm squares within an arena measuring 1.7 x 1.7 m. The testing was performed by one person over a 4 day period between the hours of 07:10 and 19:25. All other aspects of testing were repeated as described in the week 4 session.

**Tonic immobility test**

At 4,21,24,27,30, and 35 weeks, the hens were evaluated using the tonic immobility (TI) test as described by Anderson & Jones, 2012 (Anderson & Jones, 2012). At week 4, the TI test was performed immediately following the open field test using the same 32 pullets (n=12, CT; n=10, EB; n=10, WR). On weeks 21, 24, 27, and 30, the TI test was performed by one person using 72 hens balanced across three treatment groups (n=24, CT; n=24, EB; n=24, WR) over a 4 day period between the hours of 5:00 and 23:30. The birds were removed from different rooms in consecutive tests and were balanced across treatments and sessions so that each hen was selected for testing a minimum of two times and a maximum of three times throughout the duration of the study. Each hen was removed from its home pen, placed in a 20 x 12 x 20 cardboard transport box and carried into a testing room located adjacent to the rooms housing the home pens. The hen was then removed from the transport box and place on its back into a Y-shaped cradle (20.3 cm cradle surface for 4 wk old pullets; 45.7 cm cradle surface for wks 21-35) that was covered in a laboratory bench pad (VWR®, Radnor, PA, USA) and restrained for 10 seconds using one hand on the sternum to induce TI as shown in Figure 7. After TI was initiated the observer would pause to ensure TI induction had been established; if not, and the bird immediately righted itself, the TI test was restarted by placing the hen back in the cradle and

restraining it again. If TI was unable to be induced after three attempts the test was canceled and the hen was returned to its home pen. Once the observer confirmed that TI had been initiated, they exited the room and monitored the test from a viewing station located in the hallway outside of the testing room. A camera (4M AHD Analog Bullet Camera, SDC-89440BF, Hanwha Techwin, Seoul, South Korea) mounted directly above the Y-shaped cradle captured video and a DVR (SDR-B85300, Hanwha Techwin, Seoul, South Korea) located at the viewing station recorded the session. The test was completed when the bird either righted itself or 10 minutes had passed since TI induction. Variables measured included number of struggles at induction, time until righted and latency to first head movement as described in Table 3. The TI test performed at week 35 was performed as described above with the exception that it was executed over a six day period.


**Figure 7.** Image of hen in tonic state during tonic immobility testing

| Behavior | Description |
|---|---|
| Hen latency to right itself | Measured time in seconds from initialization of tonic state to the time hen breaks from tonic state and rights itself |
| Number of struggles | Number of struggles the hen performs before tonic state is achevied |
| Vocalization | Number of vocalizations produced by the hen before tonic state is achieved |
| Hen latency to head movement | Measured time in seconds from start of tonic state to first head movement |

**Table 3.** Ethogram of tonic immobility test

## Blood Sampling

Upon completion of the TI test the bird was placed back into the transport box and relocated into a hallway until a time of 15 minutes had elapsed since removal from home pen. This allowed for the corticosterone response to reach its peak (Fraisse & Cockrem, 2006; Daigle, Rodenburg, Bolhuis, Swanson, & Siegford, 2014). At that time, a 2-3 ml blood sample was taken from the brachial vain of the hen using a 21-22 gauge needle and 3 ml syringe. The sample was then immediately transferred to a purple top EDTA tube (Becton Dickinson, Franklin Lakes, NJ, USA) and placed on ice until it was centrifuged at 930g for 6 min at 4ºC. The plasma was then transferred into 2 ml cryogenic vials (Corning, New York, NY, USA) and stored at -80ºC until the day of corticosterone analyses.

## Corticosterone analysis

The collected plasma samples were removed from the freezer and allowed to thaw completely before being tested in duplicate using the Invitrogen Corticosterone Competitive ELISA kit (Life Technologies Corporation, Frederick, MD, USA). This is a solid-phase competitive enzyme-linked immunosorbent assay that detects and quantifies the level of corticosterone in plasma independent of species (Life Technologies Corporation, 2017). The

samples were run according to manufacturer instructions and the plates were read using an Infinite M200 PRO microplate reader (Tecan, Mannedorf, Switzerland) at 450 nm.

**Behavioral observations in home pen**

At week 22 through week 25 a camera (4M AHD Analog Bullet Camera, SDC-89440BF, Hanwha Techwin, Seoul, South Korea) mounted at the top of each home pen recorded hen behavior during two 30 minute periods (8:00-8:30h and 20:30-21:00h), similar to protocols used by Rodenburg and Koene (2003), as well as Daigle, Rodenburg, Bolhuis, Swanson, and Siegford (2014) (Rodenburg & Koene, 2003; Daigle, Rodenburg, Bolhuis, Swanson, & Siegford, 2014). The video footage as seen in Figure 8 was recorded using a DVR (SDR-B85300, Hanwha Techwin, Seoul, South Korea) and later reviewed using scans at 5 minute increments to determine the number of hens eating, drinking, foraging, preening, and perching (Table 4) within the 30 minute period. The same 30 minute time segments were also used to evaluate pecking behavior in the home pen (Table 5). The number of aggressive pecks (AP) and gentle pecks (GP) was recorded within the two 30 minute periods. All of the observations were made by the same person using behavioral observation research interactive software (BORIS) v.6.3.6 (Friard & Gamba, 2016).

| **Posture** | **Description** |
|---|---|
| Walk | Three consecutive steps traveled in a direction with the head of the hen moving in a lateral forward and backward motion consistant with the direction of the body |
| Stand | Hen is in an upright position with the body supported above ground by legs |
| **Behavior** | Description |
| Drink | Hen is positioned near water source with head underneath nipple using beak to drink |
| Forage | Hen holds head in a lower position than rump and pecks at substrate while moving forward or standing. Identified when the hen makes >3 successive pecks at substrate, or if in a hen is in foraging posture and has not performed other behaviors in the previous 5 s |
| Eat | Hen is postioned by the feeder and is using beak to consume grain |
| Perching | Hen is in a sitting position on either perch or water line |
| Preening | Hen is either sitting or standing while using beak to clean, pull or rearrange feathers on self |

**Table 4.** Description of home pen behaviors collected during video observation


**Figure 8:** Image from home pen camera used for behavioral observation and feather pecking behavior

| Pecking behavior | Description |
|---|---|
| Gentle feather pecking | Hen gently pecks at feathers of conspecifics using beak. This pecking does does not result in trauma or removal of feathers to the receiving bird and is most often ignored. GFP most often occurs in bouts where several gentle feather pecks are delivered to the back or tail of the recieving bird. The total number of pecks was counted. |
| Aggressive peck | Hen raises head and forcefully directs beak toward conspecific once or multiple times. These pecks are most often directed to the head, but may be delivered to the body as well. Hens receiving AFP show avoidance behavior, trying to duck or run from the aggressor. The total number of pecks was counted. |

**Table 5.** Description of gentle and aggressive feather pecking behaviors

## Hen weight and egg production

The final hen weight was recorded at week 36 (n=53, CT; n=50, EB; n=51, WR) and egg production was recorded over a 28 day period starting on week 17 and continuing through week 21.

**Statistical analysis**

Statistical analysis was used to identify any significant differences found between treatment groups in behavior testing, recorded pen behavior, CORT and egg production/final hen weight. The difference or ratio between the CF culture treatment coefficient and the other treatment coefficients is referred to as treatment effect. All of the statistical tests performed involved transformed data and cannot be interpreted on the original response scale. Results are summarized using the notation (coefficient, stdev, p-value). Exploratory analysis of the treatment effect was performed on recorded responses to reveal any visual trends and to check distribution assumptions. Statistical models were selected based off of these findings. Most models used in the analysis utilized treatment as the only fixed effect and included a random bird effect if hens were tested multiple times. For the majority of models, it was found that the treatments could not explain the variance of the response.

Exploratory analysis revealed data with overrepresented values of 0's found in count data (home pen behavior counts and behavior testing count data). Zeros were recorded to indicate the absence of an observed behavior at a specific time point and resulted in skewed data distribution. A generalized linear mixed model was used with zero-inflation to account for the large number of zeros recorded. The frequency of zeros found in the count data is larger than would be expected with a standard Poisson distribution; to account for this, a zero-inflated Poisson distribution (ZIP) regression was applied to grooming, drink, eat, forage, stand and walk responses. ZIP regression adjusts for zero-inflated data by inflating the probability of a zero from the Poisson distribution by a fixed percent.

In some responses the mean and variance differed significantly even after zeros were removed. This implies that the Poisson part of the ZIP was not a suitable fit for the data

(overdispersion) as the mean and variance should be equal for a Poisson random variable. Squares entered, steps taken, defecation, perch, preen, and feather pecking were therefore modeled with zero-inflated negative binomial regression (ZINB), which can generalize the ZIP distribution. Some responses also contained a very small proportion of extreme outliers which were removed to maintain goodness of fit.

Latency to first step responses recorded in the open-field test, hens latency to right one-self and time to first head movement data recorded from the tonic immobility test were all timed responses. This timed data was type 1 censored, meaning there is a time limit after which observations are censored. Because all of the timed responses collected in the open-field and tonic immobility tests were limited to 600 seconds, multiple 600 second observations were recorded. This created an issue similar to the one found in the count data where zero inflated responses affected data distribution. Skewed distribution made parametric models a poor fit for timed responses leading to a non-parametric approach and selection of censored survival analysis. Under this method of analysis, a Cox proportional hazard model was used to force the treatments to have the same shaped hazard functions. The functions were scaled with coefficients representing the treatment and bird random effect where required. The treatment effect is the difference between the rates at which the timed events occur. This interpretation evaluates timed responses in a fashion similar to a disease state that the hen survives from, with our focus being survival rate.

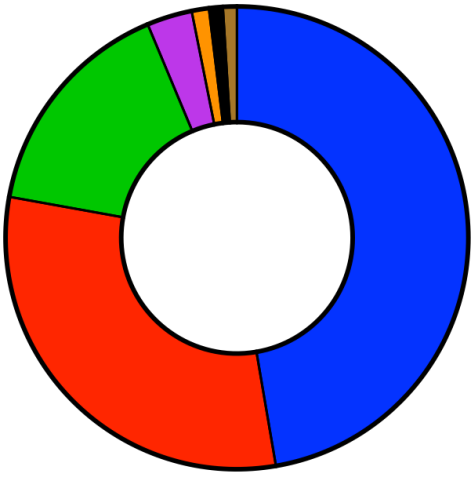
The CORT values used in statistical analysis were log-transformed and fitted with a linear mixed effect model involving treatment and week as fixed effects with bird ID as a random effect used due to hens being tested multiple times. All statistical analysis was performed using R with significance set at $p \leq 0.05$.

CHAPTER IV

RESULTS

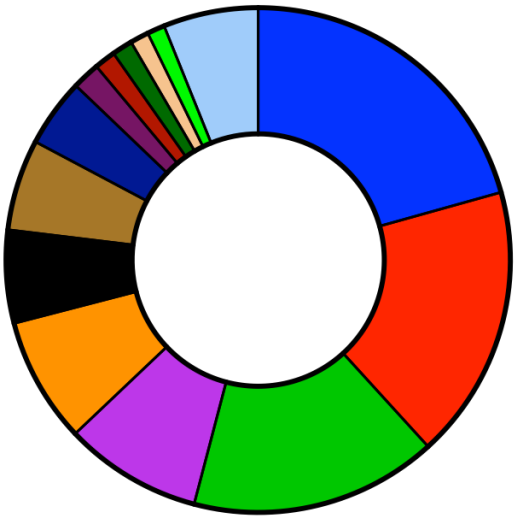
## CF culture microbial diversity

The microbial composition of the CF culture at the kingdom level displayed 96.4% of the identified reads were bacteria, 3.5% were viruses and < 1% were archaea. Of the bacteria, 218 OTUs were identified from 102,353 reads. From these OTUs, 7 bacterial classes (figure 9) were defined and 59 individual bacteria were identified at the species level. Thirteen Bacterial species composing 94% of the total CF culture are represented in figure 10 below. The remaining 6% of the bacteria (identified as "other" in figure 10) is made up of the remaining 46 bacterial species that individually only each make up <1% of the total identified reads.



| Class | Percent of Identified Reads |
|---|---|
| Clostridia | 47.30 |
| Bacteroidia | 30.53 |
| Synergistia | 15.86 |
| Erysipelotrichia | 3.16 |
| Bacilli | 1.20 |
| Actinobacteria | 0.97 |
| Negativicutes | 0.97 |

**Figure 9.** Bacterial identification of CF culture at the class level



| Species | Percent of Identified Reads |
|---|---|
| *Moryella indoligenes* | 20.67 |
| *Sporanaerobacter* spp. | 17.56 |
| *Pyramidobacter piscolens* | 15.86 |
| *Bacteroides* spp. | 8.83 |
| *Bacteroides uniformis* | 8.04 |
| *Prevotella stercorea* | 5.96 |
| *Clostridium* sp. | 5.84 |
| *Porphyromonas somerae* | 4.38 |
| *Solobacterium* spp. | 1.78 |
| *Faecalicoccus clostridiales bacterium* | 1.36 |
| *Lachnoclostridium clostridium bolteae* | 1.32 |
| *Parabacteroides distasonis* | 1.19 |
| *Rummeliibacillus stabekisii* | 1.13 |
| other | 6.09 |

**Figure 10.** Bacterial identification of CF culture at species level

From this analysis, the population of the CF culture is composed of a mix of gram positives and negatives. The majority of these bacteria are anaerobic with many being obligate anaerobes. Of the 13 bacterial species that make up 94% of the CF culture only one, *Rmmeliibacillus stabekisii* was aerobic. The bacterial profile of the CF culture at the species level reveals that more than 50% of the total identified reads were composed of 3 bacteria. *Moryella indoligenes* (20.67%), *Sporanaerobacter* spp. (17.56%), and *Pyramidobacter piscolens* (15.86%) account for over half of all the identified reads from the CF culture. At the class level, Clostridia, Bacteroidia and Synergistia account for approximately 94% of the total identified reads.

**Ebeam inactivation of CF culture**

***Inactivation of growth***

Evaluation of the EB culture was performed to determine which eBeam delivered dose effectively inactivated growth of the CF culture. The evaluation was studied by exposing the CF culture ($9 \times 10^8$ CFU/ml) to incremental eBeam doses (~2, 4, 8 and 10 kGy). Viable colony forming units (CFU) described in Table 6 were calculated from aerobic and anaerobic plate counts of the irradiated samples to identify the reduction in CFU with respect to delivered dose. Incubation of the irradiated culture was performed aerobically and anaerobically to ensure inactivation was effective in each atmospheric condition. The evaluation also contained a sample that received 0 kGy in order to evaluate the effect of collection, packaging and transport on the CF culture. Results of this evaluation revealed an approximate one log reduction due to the collection, packaging and transport and identified that 8 kGy was sufficient in inactivating the CF culture.

| **Anaerobic** | | **Aerobic** | |
|---|---|---|---|
| **Delivered Dose (kGy)** | **CFU/ml** | **Delivered Dose (kGy)** | **CFU/ml** |
| 0 | $1.98 \times 10^7$ | 0 | $2.88 \times 10^6$ |
| 2 | 66 | 2 | 0 |
| 4 | 66 | 4 | 0 |
| 8 | 0 | 8 | 0 |
| 10 | 0 | 10 | 0 |

**Table 6.** Results of Ebeam irradiation on CF culture ($9 \times 10^8$ CFU/ml)

To ensure total inactivation of the CF culture, a delivered dose of ~ 10 kGy was selected for preparing the EB culture used in this experiment. As previously discussed, the CF culture was packaged and processed at the eBeam center in batches and stored in anaerobic jars at 4°C until time of delivery. To confirm inactivity of the 100 ml EB culture aliquots at day of delivery, each unit from a batch was aerobically and anaerobically plated and the pH of the sample was measured as noted in table 5 below.

| | | Number of Hens | | Measured pH | | Aerobic/Anaerobic Plates | | | |
|---|---|---|---|---|---|---|---|---|---|
| Week | Date | Total Receiving Water | Total Receiving Ebeam | pH Water | pH of Ebeam | Ebeam Growth | Water Growth | Ebeam Session | Delivered Dose |
| 1 | 12/21/17 | 72 | 72 | 8.26 | 6.33 | NG | NG | 12/19/17 | 10.45 kGy |
| 2 | 12/28/17 | 71 | 69 | 9.19 | 6.88 | NG | NG | 12/19/17 | 10.63 kGy |
| 3 | 1/4/18 | 70 | 69 | 8.91 | 6.42 | NG | NG | 12/19/17 | 10.65 kGy |
| 4 | 1/11/18 | 70 | 69 | 7.66 | 6.45 | NG | NG | 12/19/17 | 10.82 kGy |
| 5 | 1/19/18 | 59 | 60 | 7.16 | 6.34 | NG | NG | 1/17/18 | 10.30 kGy |
| 6 | 1/25/18 | 59 | 60 | 8.34 | 6.42 | NG | NG | 1/17/18 | 10.35 kGy |
| 7 | 2/1/18 | 56 | 60 | 8.07 | 6.46 | NG | NG | 1/17/18 | 10.25 kGy |
| 8 | 2/8/18 | 56 | 60 | 8.35 | 6.39 | NG | NG | 2/6/18 | 10.71 kGy |
| 9 | 2/15/18 | 56 | 60 | 7.77 | 6.37 | NG | NG | 2/6/18 | 10.79 kGy |
| 10 | 2/22/18 | 56 | 60 | 7.04 | 6.55 | NG | NG | 2/6/18 | 10.28 kGy |
| 11 | 3/1/18 | 56 | 60 | 7.67 | 6.35 | NG | NG | 2/6/18 | 10.53 kGy |
| 12 | 3/8/18 | 56 | 60 | 8.09 | 6.1 | NG | NG | 3/6/18 | 10.44 kGy |
| 13 | 3/15/18 | 56 | 60 | 7.61 | 6.13 | NG | NG | 3/6/18 | 10.52 kGy |
| 14 | 3/23/18 | 56 | 60 | 7.34 | 6.17 | NG | NG | 3/6/18 | 10.52 kGy |
| 15 | 3/29/18 | 56 | 60 | 7.27 | 6.19 | NG | NG | 3/6/18 | 10.77 kGy |
| 16 | 4/5/18 | 56 | 60 | 7.04 | 6.22 | NG | NG | 3/6/18 | 11.31 kGy |

**Table 7.** Description of EB culture and WR at day of delivery

Table 7 also describes the pH and confirms lack of bacterial growth for each autoclaved distilled $H_2O$ sample supplied to the WR treatment group from week 1 to week 16. This was performed to ensure that the sterilization of the water was effective and that no unintended bacteria were supplied to the WR treatment through contaminated water.

### *Evaluation of EB culture cell membrane*

In order to evaluate the impact of ebeam irradiation on the cell wall of the bacteria making up the EB culture, the cell membrane integrity was assessed using the LIVE/DEAD® *Bac*Light ™ Bacterial Viability Kit. As described earlier, bacteria with an intact cell membrane

will stain fluorescent green, whereas bacteria with a damaged cell membrane will stain fluorescent red. This evaluation was performed at incremental ebeam delivered doses (~2, 4, 8 and 10 kGy) and visualized in Figure 11 below.

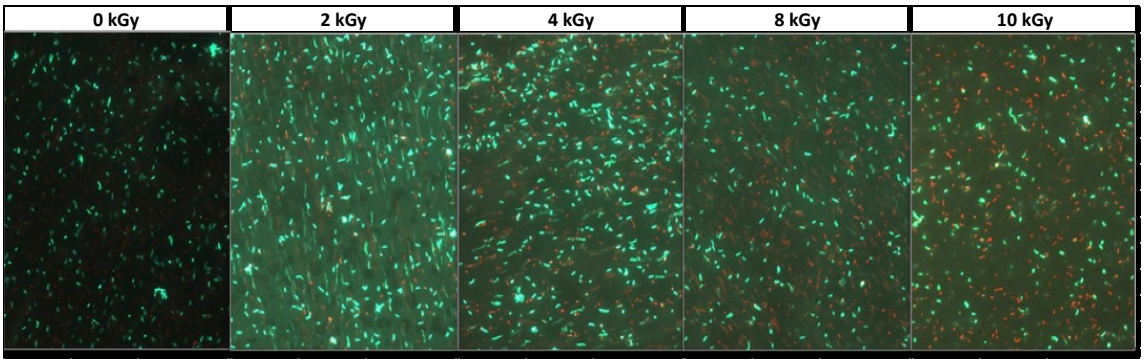



**Figure 11.** EB culture samples at incremental delivered eBeam dose visualized using *Bac*Light ™ stain.

Figure 11 illustrates that as the delivered eBeam dose increases so does the extent of cell membrane damage. While the membrane integrity of the 10 kGy sample shows the most damage as compared to the other delivered doses, it still contains approximately half of the bacterial population with an intact cell membrane.

***Metabolic activity of the EB culture***

A EB culture was prepared by collecting, anaerobically packaging and processing CF culture at the eBeam facility. The EB culture was processed at ~ 10 kGy and stored in an anaerobic jar at 4°C before being evaluated 24 hours post irradiation using alamarBlue® reagent. AlamrBlue® reagent measures enzymatic activity, which is a key indicator of bacterial metabolic activity (Davey, 2011). The processed EB culture sample was run along-side a negative control

that contained dye only, a CF culture sample that was packaged and stored in the same manner as the EB culture sample and a supernatant sample that was sourced from the EB sample (EB culture centrifuged at 5000 rpm for 10 min and filtered using a 0.22 micron syringe filter) (Millipore, Billerica, MA). The supernatant was run in order to identify the level of background noise potentially attributed to erroneous reduced compounds that may have been generated during eBeam irradiation.

The results (data not included in this report) showed that, as expected, the packaged CF culture had the highest florescence value followed by the EB culture sample. This result is consistent with the data visualized by the LIVE/DEAD® *Bac*Light ™ stain that illustrated a relationship of increasing eBeam delivered dose and elevated cell membrane damage. Therefore, this slight reduction in detected florescence could be attributed to the compromised bacterial cell wall integrity sustained from the delivered 10 kGy eBeam dose. While the florescence of the EB culture is slightly lower than the CF culture it is substantially higher than both the negative control and the supernatant sample providing evidence that the bacteria comprising the EB culture is still metabolically active following ebeam processing at 10 kGy.

**Hen weight and egg production**

Hens from each treatment group (n=53, CT; n=50, EB; n=51, WR) were weighed at week 36 in order to evaluate any differences in final weight (Figure 12). No significant difference in final hen weight was detected between treatments. Egg collection data was also evaluated during a 28 day period from week 17 through 21 and is described in Figure 13 and 14. This data also

showed no significant difference in the number of eggs produced between treatments during this period.

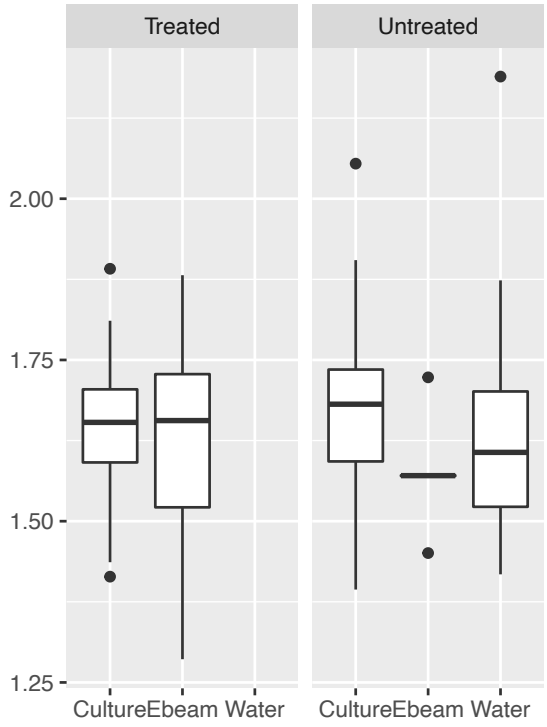



**Figure 12.** Hen weight. The treated and untreated columns identify hens with trimmed beaks (Treated) and untrimmed beaks (Untreated)

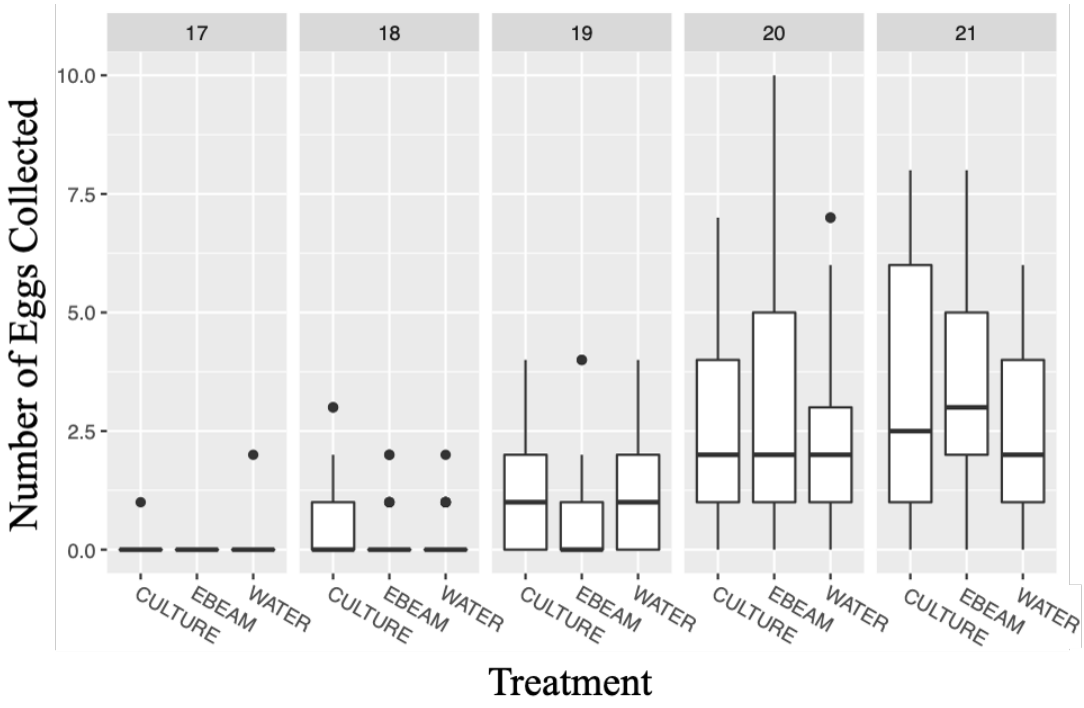



**Figure 13.** Number of eggs of collected over a five week period

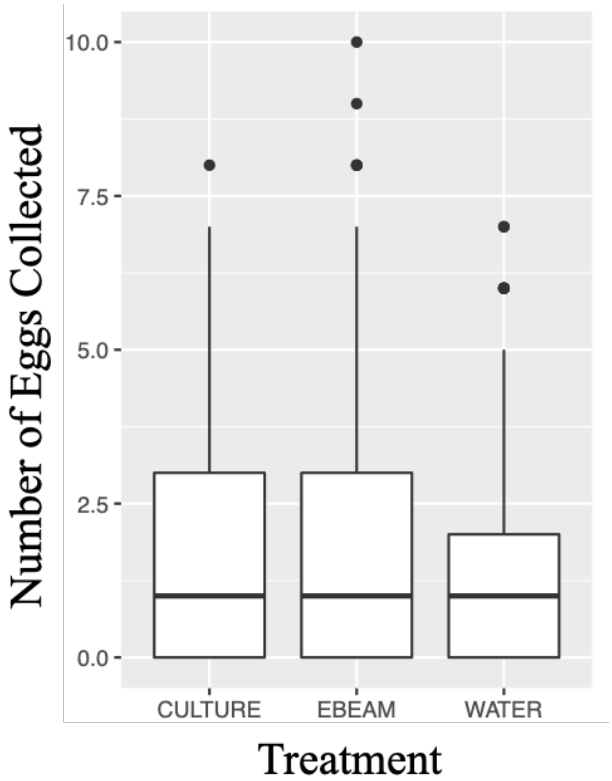



**Figure 14.** Number of eggs collected over all weeks

## Corticosterone

CORT was measured in the hens to evaluate a physiological change in response to stress. The three treatment groups were evaluated to see if any particular treatment had an impact on this response. Whole blood samples were collected from the hens after completion of the tonic immobility test and kept on ice until they were centrifuged, the plasma removed and stored at -32°C. At the time of CORT testing plasma samples were thawed and run in duplicate using the Invitrogen Corticosterone Competitive ELISA kit and read using an Infinite M200 PRO microplate reader. The data was evaluated and duplicate results with a coefficient variation of less than 20% were averaged and used for statistical analysis. Figure 15 below plots the median CORT values for each treatment during weeks 21, 24, 27,30 and 34 and figure 16 plots the median for each treatment over all weeks. A linear mixed effect model involving treatment and week as fixed effects with bird ID as random effect showed that there were no significant differences in CORT levels between treatment groups.

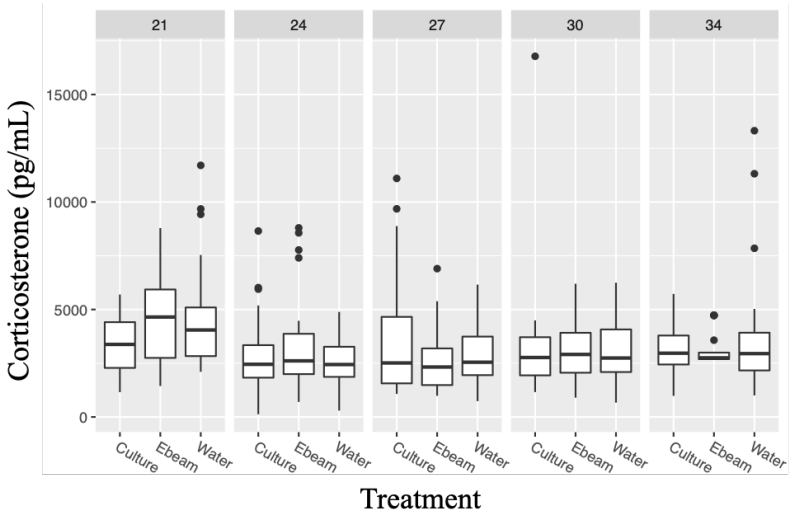



**Figure 15.** Median Corticosterone by week

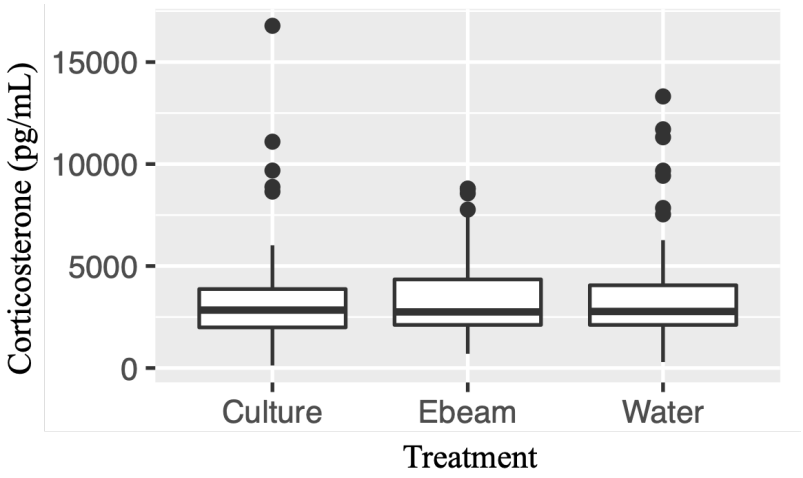



**Figure 16.** Median Corticosterone for all weeks (21, 24, 27, 30, 34)

**Open-field test**

The open-field test is routinely used in the study of emotional reactivity and motivation in poultry and lab animals (Rodenburg, et al., 2003). The behaviors observed during the open-field test have been proposed to be a compromise between opposing tendencies for the hen to avoid detection by predators and a desire to return to conspecifics in the home pen (Gallup & Suarez, 1980). Latency to first step, as well as the number of squares entered, steps taken, defecations and preening events were all behaviors evaluated in the open-field test. Latency to move, number of squares entered, and the number of steps taken are good measures of ambulation and most indicative of fear (Forkman, Boissy, Meunier-Salaun, Canali, & Jones, 2007). There is also an established relationship between the open-field test and feather pecking behavior (Jones, Blokhuis, & Beuving, 2007).

No usable data was garnered from the open-field test performed at week 4. All of the subject hens remained in a frozen state within the starting square of the arena for the totality of the test. For this reason, week 4 data was omitted and only week 35 data was used in this

analysis. The squares entered, steps taken, defecation and grooming data sets all contained more zeros than would be expected with a standard Poisson distribution. Because of the zero-heavy data, a zero-inflated Poisson (ZIP) distribution was used for number of squares entered, steps taken and defecation. ZIP regression models a fixed percent of the responses as zero with the remaining responses following a standard Poisson distribution. Even with the removal of zeros, the mean and variance of the grooming data set differed significantly, implying that the Poisson portion of the model did not fit the data. A zero-inflated negative binomial (ZINB) regression was used for this data, which generalizes the ZIP distribution. No significant differences were found between treatments in any of the observed behaviors recorded during the open field test.

The data associated with latency to first step in the open-field test was type 1 censored that required a nonparametric approach. Survival analysis was selected for interpreting this data with timed responses being treated like "diseases" that the chicken "survives" from. This allows for the evaluation of survival rates between treatments. Figure 17 below describes the proportion of hens in each treatment group that have yet to make an initial step by the time shown on the x-axis. No treatment showed any significant differences in latency to first step in the open-field test however, the water treatment group did near significance (-0.75, 0.47, 0.09).

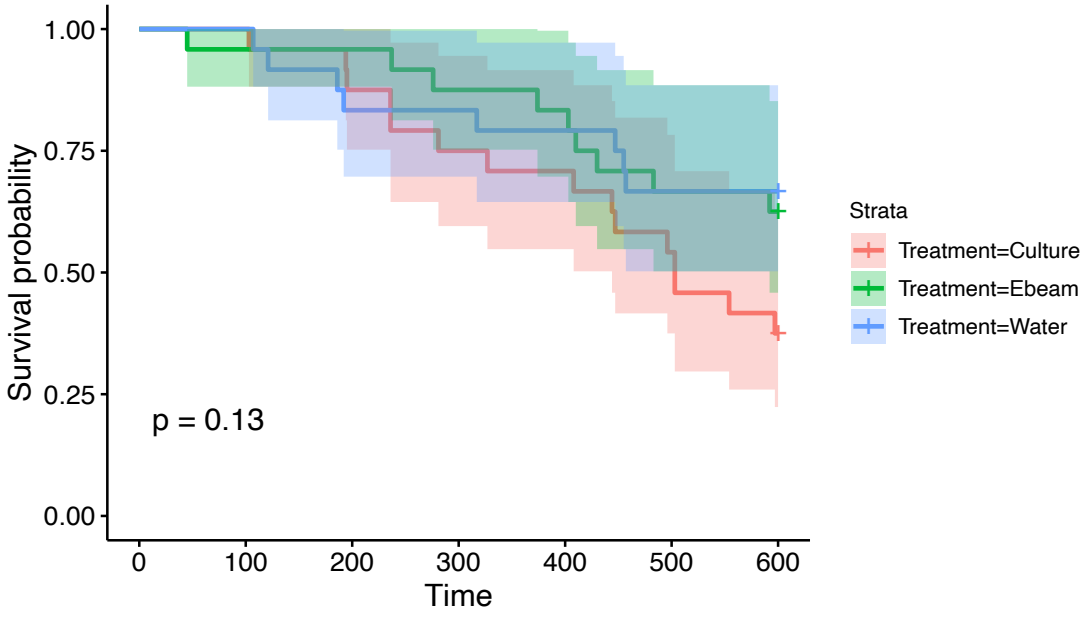



**Figure 17.** Latency to first step. The shaded areas represent confidence intervals along the curve

**Tonic immobility test**

Tonic immobility is a test that measures an unlearned hen response characterized by paralyzed or freezing behavior in response to external stimuli. This test is commonly considered a measure of fear with longer freezing states associated with higher levels of fearfulness (Wang, et al., 2014). Variables measured within the test includes the time it takes for the hen to right it-self after tonic state has been established (hen latency to right itself), number of struggles the bird performs before a tonic state can be achieved (number of struggles), number of vocalizations performed immediately before and during initialization of tonic state (vocalizations) and time to first head movement after establishment of tonic state (hen latency to head movement). As with the open-field test, week 4 results were omitted from analysis due to unresponsiveness in the hens to testing.

Results for the number of struggles and vocalizations included many zeros affecting the distribution of the data. ZIP regression was used to analyze this data and no significant differences between treatment groups were found in the number of struggles or vocalizations during the tonic immobility test. The data associated with hen latency to right itself and hen latency to head movement were type 1 censored and analyzed with the same nonparametric censored survival model described in the open-field test. Figure 18 below shows the proportion of chickens that have not yet righted themselves by the time shown on the x-axis. The culture treatment group represented with the red line is higher than the eBeam and water treatment groups suggesting the rate is slightly lower, and that treatment causes hens to take a longer time to self-right. While this effect is noticeably different for the culture treatment group it was not significant given the sample size according to the p-value.

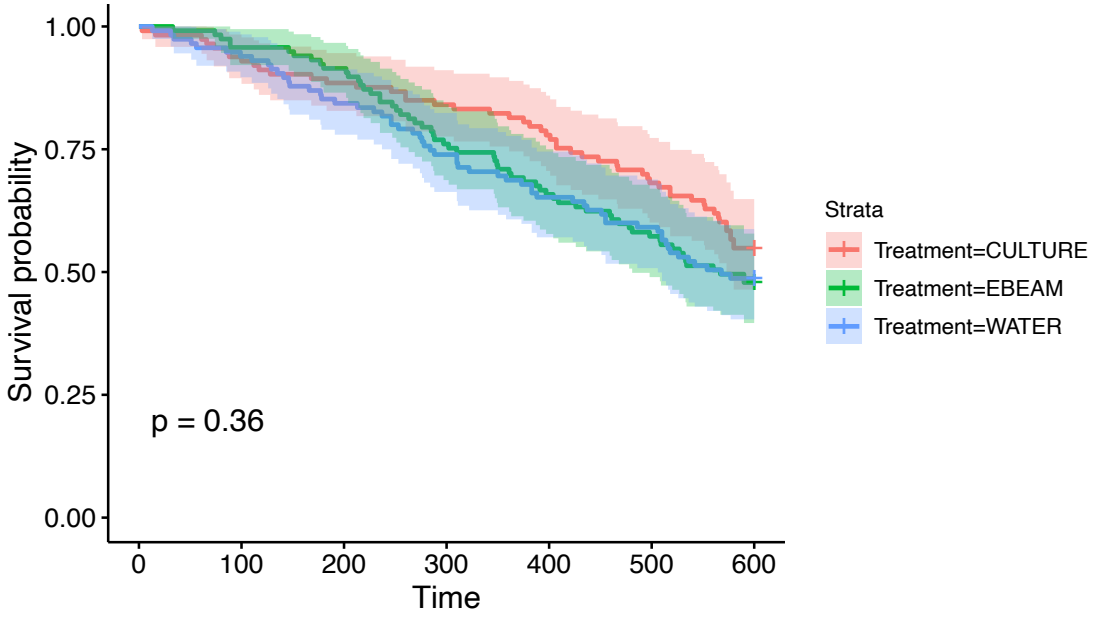



**Figure 18.** Hen latency to right itself. The shaded areas represent confidence intervals along the curves.

Latency to head movement was evaluated in the same manner as hen latency to right itself. Figure 19 below plots the rates for each treatment with no significant differences found. We find that the rates for head movement are essentially identical between treatments.

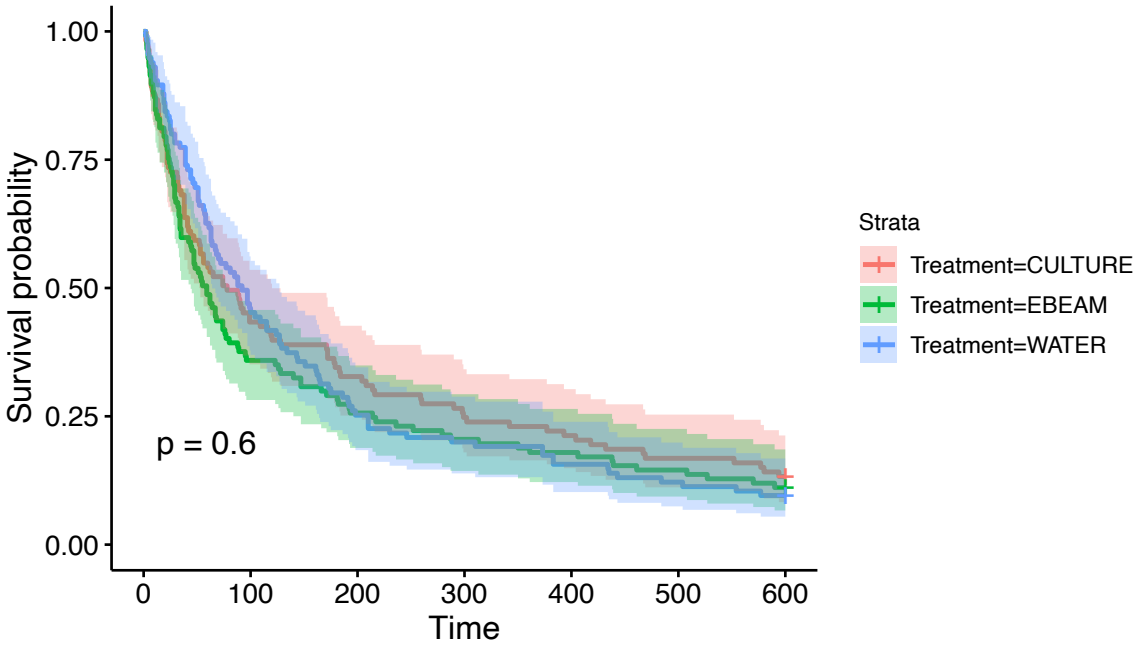



**Figure 19.** Hen latency to head movement. The shaded areas represent confidence intervals along the curve

**Home pen behavioral observations**

On week 22 through 25 the behaviors performed by hens in their home pens was recorded. This data was counted and the count-based matrices were used in analysis. As with the data sets from the open-field and tonic immobility test, much of the home pen behavioral observational data was also heavily influenced by the presence of multiple zeros. A generalized linear mixed model with zero inflation was used, and depending on dispersion, either a Poisson or negative binomial distribution was selected.

Some of the observed behaviors such as dust bathing, rest and sit contained so many zeroes that the treatment effects were not significantly different from 0 and, as such, were not used in analysis. Figure 20 below includes perching, eating, foraging, preening, walking, and standing behaviors, which all had significant non-zero means but showed no significant treatment difference. The only significant difference found in the observed pen behaviors was drinking (0.28, 0.12, 0.02), which was performed significantly more often by the WR treatment hens than hens receiving CT or EB cultures.

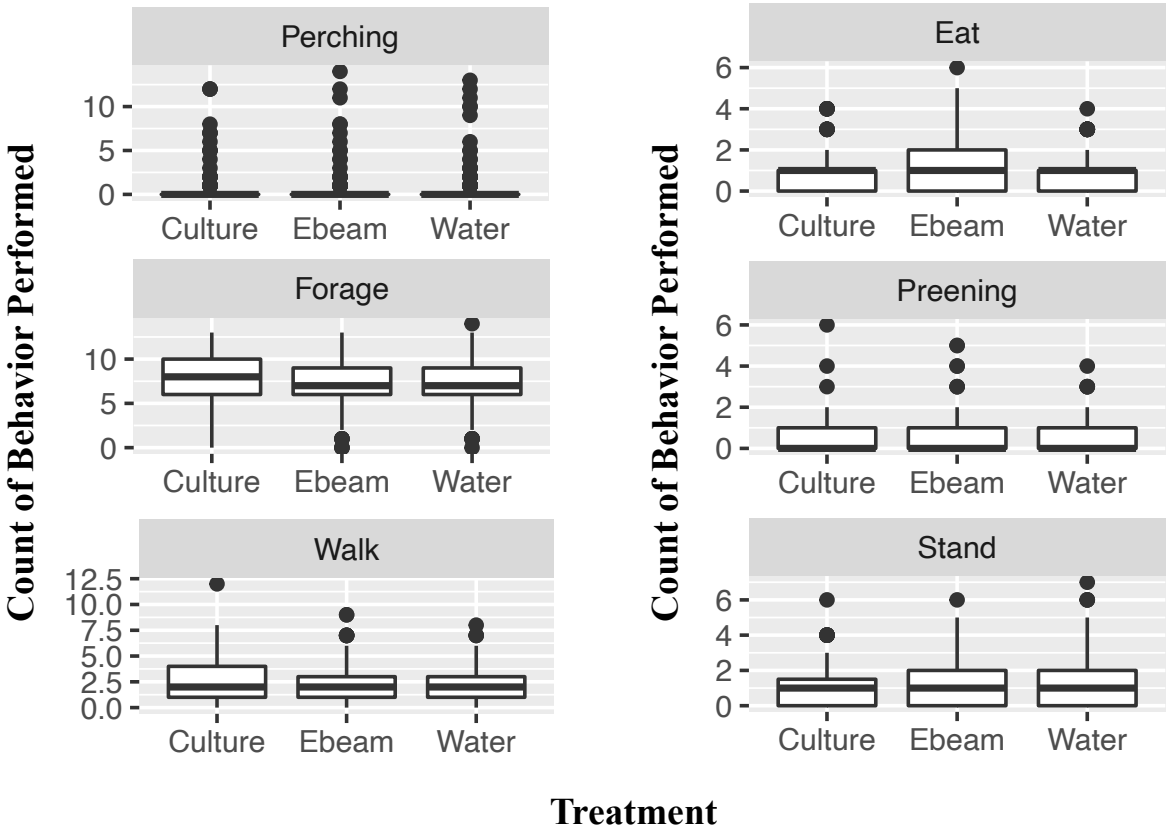



**Figure 20.** Description of pen behaviors from week 22 – 25

Feather pecking behaviors were observed and documented during the same time segments as the home pen behaviors. The data set associated with aggressive feather pecking

had a significant non-zero mean but displayed no significant difference between treatments. Data associated with gentle feather pecking contained so many recorded zeroes that the treatment effect was not significantly different from zero. This limits the interpretation of the data because the number of recorded zeroes associated with any one treatment could be larger due to coincidence thereby affecting the detection of any valid differences attributed to treatment. With this in mind, the WR treatment group showed significance ($p = <1e\text{-}6$) with respect to the number of gentle feather pecks delivered as compared to the CT and EB groups. While this analysis points to a difference between treatment groups, it must be evaluated within the proper context as it is most likely a spurious result produced by an inflated number of zeroes being introduced into the data set. This result requires a larger study in order to confirm any significant effects.

# CHAPTER V

# DISCUSSION

The approach taken by this study to reduce aggressive feather pecking behavior in laying hens was to establish a "healthy" gut environment colonized by commensal bacteria supplied through a CF culture. The conceptual framework surrounding this idea involved the introduction of beneficial bacteria early in hen maturation to assist with the development of biological systems that could potentially shape the behavior of the hen throughout its lifespan. It also included an irradiated treatment prepared using the same CF culture to investigate the potential impact of non-colonizing bacteria on feather pecking behavior.

Early colonizing "healthy" commensal bacteria are key players in immune modulation, nutrient processing, cognitive development, and stress response. These bacteria are usually derived from the environment, feed source and exposure to adult animals. Probiotic supplementation as utilized in this context is a strategy of providing early bacterial exposure to post-hatch chicks in the absence of healthy adult conspecifics. Numerous studies provide evidence of cognitive and behavioral changes in animals attributed to the administration of probiotics. *Lactobacillus rhamnosus* was found to reduce stress-induced corticosterone and anxiety- and depression-related behavior in mice (Bravo, et al., 2011) and *Bacteroides fragilis* has been shown to correct gut permeability, reduce anxiety and alleviate behavioral abnormalities found in maternal immune activation mice (Hsiao, et al., 2013). While these examples show the significant influence of a probiotic composed of a single bacterial species to ameliorate dysbiosis or gut deficiencies, a slightly different approach must be used to establish a beneficial gut microbiome at day of hatch. Ecologically it is hypothesized that a healthy host

microbiota resembles the microbiota transferred from healthy parents to their offspring (Marcolla, Alvarado, & Willing, 2019). This would indicate that a consortium of bacteria sourced from the gut of a healthy adult animal would be ideal for use as a probiotic in establishing early gut colonization. The CF culture used in this experiment was selected with this in mind. It was constructed using identified bacterial species sourced from the cecal contents of a 6-week-old healthy pig. While the CF culture was designed using a microbiome that originates from a different species, comparisons between the gut environment and bacterial populations of the two animals show many similarities making it a good choice for this study (Genovese, et al., 2003).

The microbial profile of the CF culture was determined using 16s rRNA sequencing. Results of this sequencing showed that 92% of the CF culture was composed of Clostridia, Bacteroidia and Synergistia at the class level, with almost half (47.3%) being Clostridia. Ding et al., 2017 found through a comparison of three different chicken breeds that the most common phyla found in maternal hens and chicks were Firmicutes (44%) and Bacteroidetes (24%), this was consistent with the bacterial make-up found in the CF culture which contained 52.6% Firmicutes and 30.5% Bacteroidetes. At the species level, *Bacteroides uniformis* and *Parabacteroides distasonis* were identified in the CF culture. Both of these bacteria, either individually or as a part of a consortium, have been used as probiotics in the past (El Hage, Hernandez-Sanabria, & Van de Wiele, 2017; Wang, et al., 2019). *Bacteroides* spp. and *Clostridium* sp. were other bacteria associated with probiotics that were identified in the CF culture supporting its use as an appropriate candidate for early gut colonization.

Hsiao, et al., 2013 described beneficial properties of *B. fragilis* without colonization in a mouse model. In order to explore the effects of a non-colonizing probiotic culture on developing

hens an irradiated version of the CF culture was created. Electron beam technology was used to prepare the EB culture using a minimum deliver dose of 10 kGy. This dose was determined by evaluating different delivered doses for growth under aerobic and anaerobic conditions as displayed in table 4. The minimum delivered dose calculated on table 4 actually indicates 8 kGy as the minimum dose required to inactivate growth, however 10 kGy was selected to ensure bacteria were unable to repair damages induced by the radiation.

Inactivation of growth was not the only parameter measured while evaluating the appropriate administered dose. The integrity of the bacterial cells within the culture must also be taken into consideration. Cell wall integrity was evaluated using the LIVE/DEAD® *Bac*Light ™ assay. Results visualized in figure 11 illustrate that approximately 50% of the bacterial cells constituting the EB culture have compromised cell walls. This highlights a potential bias introduced through ebeam processing. The metagenomic data identified 59 individual bacteria within the CF culture, each of these bacteria have unique D-10 sensitivities. As discussed in the literature review, D-10 values are a measure of specific bacterial resistance to ionizing radiation. They provide information on the dose required to achieve a 90% reduction in the number of viable microbial cells. This value helps calculate a delivered dose that is high enough to effectively inactivate propagation while low enough to preserve the integrity of the cell wall and metabolic function of the bacteria. Each of the 59 bacteria have their own D-10 value and a uniform delivered dose of 10 kGy may be too high for some of the bacterial species included in the culture to maintain structural integrity. This observation was supported by the results obtained through the Alamar blue assay that measured metabolic activity of a 10 kGy treated EB culture sample along-side a CF culture sample. While the EB culture sample did show considerably more florescence than the negative control and supernatant, its detected florescence

was less than the CF culture sample, suggesting a decline in viable cells capable of reducing resazurin to resorufin. Overall, this means the EB culture used in this experiment was more of an inactivated subset of the CF culture, containing only a percentage of the unprocessed CF cultures metabolically active bacteria. This acknowledges an area of future exploration into potential processing techniques that can effectively inactivate mixed bacterial cultures while maintaining cell viability throughout.

Following the selection of the CF culture and development of the EB culture the treatments were delivered to chicks. The chicks were procured from a hatchery at day of hatch and received their first gavage of treatment (CF, EB or WR) while being placed in home pens. The facility housing the hens was separated into 6 individual rooms, each room included 3 pens (1 pen of each treatment) as described in figure 2. The initial placement of birds was performed treatment by treatment, meaning all of the hens receiving CF culture were gavaged and placed in the appropriate pens, then the EB group and finally the WR. This was significant because on week 8 the condition of the cardboard brooder board at the base of the pens was noticeably different between treatments. The brooder boards found in the WR treatment group pens were ripped and torn with many holes, while the boards contained within the CF treatment group pens were in much better condition, showing fewer rips, tears and holes. The boards contained in the EB treatment group pens were in the best condition of all, sustaining very little damage. This observation lead to the discovery that some chicks had received beak modification at the hatchery and others had not. Because of the original method used to place the chicks, the EB treatment group contained almost all beak modified birds; the CF group contained a mix of modified and unmodified beaks, and lastly the WR group didn't contain any modified birds at all. The conditions found in the brooder board were a result of more hens assigned to the EB

treatment group receiving beak modification than in the WR treatment group. This unfortunate oversite could have introduced bias into the study with regard to home pen behavior. The only home pen behavior that displayed any significant difference between treatments was found in the number of drinks performed by the WR group (p=0,02). This result could be influenced by beak modification as hens with modified beaks may experience a loss of sensitivity due to severed nerves of the beak (Freire, Eastwood, & Joyce, 2011). Loss of beak sensitivity, function or discomfort while drinking may affect drinking efficiency from the water apparatus, which may explain decreased trips to the water nipples by the CF and EB groups. Hen weight between treatments showed no significant differences and eating behavior counts were unremarkable, suggesting beak alteration had little influence on the hens ability to consume feed. With regard to feather pecking behavior however, research examining beak modification with relation to feather pecking behavior has shown that there is no evidence that beak alteration affects feather pecking behavior, but rather reduces the feather damage sustained through this behavior (Nicol, et al., 2013). Furthermore, exploratory analysis performed on aggressive feather pecking behaviors between treatments was evaluated using week, room and pen as covariates. This was performed to investigate deficiencies in experimental design and identify any possible bias they may have introduced into the study. The analysis showed that over the observed 4-week period; week, room and pen had no effect on aggressive feather pecking.

The behavioral tests used in this study were performed to identify any differences in fear response. This change in fear response could indicate an exaggerated stress response potentiated by early HPA axis programming. Analysis of the open-field and tonic immobility tests showed no differences between treatments. These observations suggest that stimulus induced activity is similar between treatment groups, meaning HPA functionality and stress response were

proportional between groups. These findings were supported by CORT analysis which also found no significant difference between treatments indicating a similar physiological HPA response to external stimuli. It is interesting that the WR group was weakly significant in latency to first step within the open-field test, however this small effect was not mirrored in the CORT data. Taken together, this analysis leads to an assumption that early development and programming of the HPA axis was not unique in any one treatment group used in this study.

Review of home pen behaviors displayed little evidence for treatment effect. All of the observed behaviors with the exception of drinking showed no significant difference. There was also no difference between groups in the number of aggressive feather pecks performed during this time period. While a significant difference was detected in gentle feather pecks delivered by the WR group, the validity of this result remains in question due to the number of zeros potentially influencing the data. It needs to be noted that the WR treatment group approached significance in standing ($p = 0.07$) behavior and latency to first step ($p = 0.09$) in the open-field test and was the only group to display significant differences in drinking behavior and gentle feather pecking. This is curious and suggests that there may be a difference between the WR treatment group and the two culture groups, but this evidence is inconsistent and further testing is required to justify this claim.

Overall, the treatment of CF culture administered to hens during the first 16 weeks does not reduce aggressive feather pecking behavior, change home pen behavior or reduce stress in behavioral tests in a significant way across measured metrics in comparison to EB or WR. This study did however produce inconsistent evidence that supports further investigation into a possible difference between the water group and the two culture groups.

Resolving aggressive feather pecking behavior through the use of a probiotic is a challenging objective. Many elements influence this behavior and understanding how those components interact to shape a behavioral response is critical in developing strategies to reduce it. This research was performed to better understand how the gut microbiome can shape behavior and explore elements of gut brain communication. A fundamental understanding is imperative for developing future strategies in therapy, prevention and evaluation of behavioral disorders that reach far greater than commercial poultry systems.

# CHAPTER VI

## SUMMARY AND FUTURE RESEARCH SUGGESTIONS

In order to further explore a potential difference between the WR group and the CF and EB groups additional research must be performed. This initial study provides a jumping-off point, laying the groundwork for future decisions shaping the concepts and direction of subsequent research. Throughout this study there were many noted observations that could be applied to future experimental designs in order to more clearly explore the impact of CF and EB cultures on feather pecking behavior.

One of those observations would be to explore the impact of the CF and EB cultures on mature hens in order to establish a baseline and investigate any effect they may have on the behavior of birds with a fully developed HPA axis. This evaluation would resemble drug trials and provide information about behavioral changes attributed to the applied cultures and their effectiveness as probiotics. Gut microbial profiles could be evaluated before and after administration of the cultures to explore changes in an already established gut and the potential of the cultures to colonize within it. This information would be helpful in describing the CF culture as a beneficial probiotic and, along with its description as a competitive exclusion culture, would strengthen its selection for day of hatch use in establishing a healthy gut microbiome.

Beyond this, a more thorough analysis of the effects of the CF and EB cultures may be required to fully understand their impact on behavior. Gut profiles are necessary to evaluate differences between treatment groups. This evaluation needs to be performed both early and as the hen matures to identify the profiles associated with each treatment group during

development. A comparison should be made within each treatment group as well as between treatment groups to identify any profiles that are unique to a treatment. This data will be critical when exploring any behavioral differences found through observation or testing.

Molecular testing of hen tissue and the CF and EB cultures could also provide useful information. Transcriptomics would provide a clearer picture of HPA axis activity following behavioral testing such as tonic immobility. Identifying gene expression in the hypothalamus and adrenal glands could provide insight into minute differences attributed to treatments in stress response and potentially reveal clues that could be helpful in subsequent research. Metabolomics would be useful in identifying metabolites found within the CF culture and offer a deeper understanding of the total composition of the supplied culture. It also provides a method for comparing metabolites between the CF and EB cultures.

Another caveat of this experiment that needs to be addressed is the delivery method of the treatments. In the original study design, treatments were to be delivered throughout the length of the study, however, by week 16 it was apparent that delivery of the treatment through gavage at this age could potential injure the birds. Oral gavage also required repeated handling of the hens week after week, which could habituate the birds, influencing their performance in behavioral testing. If supplementation of the treatments is to be administered throughout the length of future studies, a different delivery method should be considered.

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