ABSTRACT

Title of Thesis:	THE EFFECT OF TRYPTOPHAN AND PROBIOTIC TREATMENT ON BEHAVIOR AND PRODUCTION PARAMETERS OF LAYING HENS
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Dietary supplementation can impact behavior expression through microorganism's activity in the gut microbiome and influence productivity in animal husbandry. Adding supplements to production animals' diet can impact behaviors and productivity via gut-brain axis activity. We investigated the effects of either probiotic or antibiotic supplementation in addition to the effects of additional tryptophan (Trp) on overall behavior and production parameters. White Leghorn chickens (n=12) were supplemented with six dietary treatments in a 2 x 3 factorial design: probiotics (1 x 10^9 CFU/L) or the antibiotic erythromycin (125 mg/L) in combination with either normal (0.16%) or high (0.48%) Trp in drinking water. Results indicate that probiotics with tryptophan increased locomotion (P = 0.04), social (P = 0.04), and eating behavior (P = 0.02). Antibiotics with tryptophan increased fat pad (P = 0.04) and heart weight (P = 0.04). Supplementation affected behavioral expression of

normal, comfort, and pecking behavior, potentially impacted by metabolic competition at the level of the gut microbiome.

THE EFFECTS OF TRYPTOPHAN AND PROBIOTIC TREATMENT ON BEHAVIOR AND PRODUCTION PARAMETERS OF LAYING HENS

by

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Dedication

I want to dedicate this thesis to my family, especially to my mother, Brenda and grandmother, Mami Luz who have stayed on the other side of the phone giving me all the support and love I needed while going through this process. Also, I dedicate my thesis to my partner, Mark. Without his unconditional patience, help, and support, I would not have reached my goal. A special dedication to my grandfather, Atún, for everything. Lastly, I dedicate my thesis to future farmers and producers from my homeland, Puerto Rico. May we continue to set the path towards being more in tune with the food we consume and to never lose the love for agriculture.

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ACTH: Adrenocorticotropic hormone

BBB: Blood-brain barrier

BW: Bodyweight

CNS: Central Nervous System

CORT: Corticosterone

DA: Dopamine

EC: Enterochromaffin cells

ENS: Enteric Nervous System

EP: Epinephrine

FCR: Feed conversion rate

FP: feather pecking

HPA: Hypothalamic-pituitary-axis

GBA: Gut-brain-axis

GF: Germ-free

GFP: Gentle feather pecking

GI: Gastrointestinal

LNAA: Large neutral amino acids

NA: Noradrenaline

NE: Norepinephrine

SCFA: Short chain fatty acids

SD: Skip-a-day

TPH: Tryptophan hydroxylase

Trp: Tryptophan

5:HT: Serotonin

5-HT1A/B: 5-hydroxytryptamine receptor 1A/B

Chapter 1: Behavioral Neurophysiology, Gut Microbiome Interactions, and their Implications on Animal Welfare

Animal welfare and poultry production

Animal production is a global economic contributor, whether through direct food production or other animal-derived products. However, increase in demand during the last several decades presents challenges to the industry. The agricultural industry has prioritized production with little thought to animal welfare, increasing their stocking densities and technological advances to facilitate management and maximize efficiency (Appleby et al., 2004). However, some of these practices such as high stocking density, confinement, heat stress, beak trimming, and handling and transportation have the potential to compromise animal welfare (Broom, 2000). During the 1960s, consumers began to question the intensive rearing methods in animal husbandry due to their correlation with poor animal welfare (Appleby et al., 2004). Ruth Harrison's work, Animal Machines (1964), emphasized the detrimental aspects of animal production and the importance of implementing better methods, and was one of the first publications to bring these issues to light (Broom, 2014).

Following the increased concern of the segments of the public about animal treatment, the Farm Animal Welfare Council was established in 1979 (UK). This group developed The Five Freedoms of Welfare as a standard to help define the animals' necessities and apply them to animal practices. These Freedoms are the freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury, or disease, freedom to express natural behavior, and freedom from fear and distress

(FAWC, 1992). Although these freedoms quickly became a valuable tool for animal scientists and producers by providing a framework to follow, multiple challenges surfaced. When applying the freedoms, it can be challenging to find solutions suitable for all production animals (Mason and Mendl, 1993). Therefore, focus on an animal's natural behavior, subjective emotional state, and biological functioning can offer a well-rounded analysis of the animal's current state that goes beyond the Five Freedoms of Welfare (Fraser et al., 1997).

Analyzing the expression of natural behaviors is ideal to obtain an overall understanding of the animal's state at the moment of study (Gonyou, 1994). However, not all natural behavioral displays indicate a positive welfare state, they can also indicate an adverse reaction to the animal's environment, health, or subjective emotions (Fraser et al., 1997). Quantifying the weight of subjective emotional states can make the observer fall into anthropomorphic assumptions (Fraser et al., 1997; Dawkins, 2008). Although emotions like fear, distress, and excitement are unequivocally shared between different species, drawing boundaries is crucial to avoid attributing human emotions to animals.

Expression of emotions requires spatial memory and adaptation, where an animal recognizes the situation and acts upon it depending on its emotional reactivity (Désiré et al., 2002). It also involves behavioral changes and endocrine responses (Désiré et al., 2002). In a biological context, solely trying to meet welfare needs through the animal's biological functioning or health parameters limits the scope of analysis. An animal's health is not the only indication of well-being, but it is often perceived as such (Humphrey, 2006). Therefore, these three methods of studying animal welfare (natural behavior, subjective feelings, and biological functioning) offer a more comprehensive analysis when considered collectively. In a scientific context, reliable data can be obtained by how the animal has adapted to changes in its environment through physiological changes (Veisser and Boissy, 2007). Therefore, a practical tool used to analyze an animal's well-being is through combining behavioral patterns and physiological functioning, such as fearful behaviors and physiological expression of stress. Implementing this practice to production systems can favorably impact welfare initiatives.

Poultry industry: its history and challenges

Egg consumption is ubiquitous in most US households. Eggs were a vital food source during precarious times in history when food and resources were scarce. With time, the rearing methods of laying hens shifted, bringing drastic changes to eggs' marketing and consumption (Mottet and Tempio, 2017). During the 19th century, eggs were a staple in US households, generally reared as backyard flocks. Post-WWI and following the surge in urbanization, the backyard flock tendency was displaced as more focus was given to production systems that could bring eggs to the market (Kidd and Anderson, 2019). This transformation occurred steadily, and in 1930, mechanization was introduced to increase efficiency. The increase in population during the 1960s led to more extensive and more integrated systems that were low in production costs and enhanced productivity (Gerber et al., 2007). These chains of events transformed the way humans consume eggs, now reaching a point where they are an indispensable product in the daily life of modern society.

The rearing of poultry for the production of eggs and meat is a global practice that provides profit to many countries (Gerber et al., 2007). Eggs are an excellent protein source for food-insecure communities or populations with low socioeconomic status, and they benefit these groups due to their capacity to provide essential nutrients at a low cost, and low consumption manner. When impoverished areas have access to nutrient-rich food products, it increases their food security (Mottet and Tempio, 2017). The expansion of the poultry production industry provided economic opportunities to developing countries.

Bessei's (2006) review exposed that intensive production systems are mainly composed of lack of enrichments, light manipulation, and stocking density. Additionally, beak trimming is a production practice aimed to reduce the onset of cannibalism and feather pecking, but at the expense of causing pain to the birds involved (Guesdon et al., 2006). The high stocking density of animals has made it possible to produce a better yield in farms but at the expense of impaired movement, increased disease transmission, and high stress levels of the animals (Asebe et al., 2016). However, producers face many obstacles along the way, including juggling commercial demands and the public's negative opinions of their rearing methods. Not every commercial producer has the resources to reallocate towards expensive changes for direct welfare improvements, however, research on alterations to the animal's diet may provide a relatively inexpensive tool to improve welfare.

The use and implications of antibiotics administration in rearing systems

The discovery of antibiotics was a groundbreaking advancement with an enormous impact on public health and veterinary medicine. Human life expectancy increased by the prevention of severe infections (Davies and Davies, 2010). With the increase of stocking density and the fast-paced production chain, antibiotics became an essential aspect of animal husbandry. Antibiotics provided the opportunity to treat large numbers of animals quickly, prevent the rapid spread of disease, and facilitate management practices. In addition, antibiotics became an efficient growth promoter for production of meat birds, providing more yield (Kirchhelle, 2018). Therefore, these drugs rapidly became the norm in animal production in therapeutics and subtherapeutic forms.

Antibiotic use has become concerning since their overuse poses a problem of antibiotic resistance to the animals medicated and potentially transferring that resistance to humans (van den Bogaard et al., 2002). The administration of antibiotics, as both a treatment against infection and as a growth promoter, also negatively impacts the balance of the gastrointestinal (GI) integrity (Iizumi et al., 2017). Recently, stricter standards have been applied to the use of antibiotics in animal production. Many of the antibiotics commonly used in animal productions were withdrawn, and their administration was only permitted under veterinary supervision (FDA, 2017).

Knapp et al.'s (2017) review indicates how the excessive use of antibiotics has caused certain bacterial strains to become resistant to antibiotics. Additionally, in a study by Garofalo et al. (2007), specific genes of antibiotic-resistant strains of bacteria were found in raw pork and chicken samples. The overuse of fluoroquinolones has caused the gram-negative bacteria, *Campylobacter*, to become resistant when infections reoccur (Hayes et al., 2004). Resistance can occur through management practices and also has an underpinning heritability aspect. Multiple countries, including the US, have therefore banned or restricted the utilization of antibiotics in animal production (Casewell et al., 2003; Anadón, 2006).

Antibiotics negatively affect the homeostasis of the gut microbiome, creating an environment of increased competition between microorganisms (Dudek-Wicher et al., 2018). In poultry, antibiotics significantly alter the composition of their microflora (Engberg et al., 2000; Torok et al., 2011). When comparing chicks fed a basal diet with antibiotics and a basal diet without, their body weight increased with penicillin, but their gut weights and lengths were reduced (Coates et al., 2007). Changes in morphology result in the impairment of the animal's ability to absorb nutrients (Xu et al., 2003), potentially impacting the animal's well-being and physiological processes. For this reason, the study of antibiotics alternatives has been underway.

Probiotics as enhancer for poultry performance

Antibiotic-free products have increased in popularity for consumers in recent years (Michaelido and Hassan, 2010; Teixeira et al., 2021). For this reason, research for alternatives to antibiotics has increased. This substitution must suffice the market's demand regarding weight and quality of meat while maintaining low consumer costs and reducing the proliferation of pathogenic infection in production animals. Prebiotics, which are non-digestible carbohydrates, have been a subject of study due to their potential capacity to enhance the growth of beneficial bacteria in the GI tract (Griggs and Jacob, 2005). Other forms of supplementation like organic acids and prebiotics have a growth-promoting effect, as studies with broilers indicate a positive effect on body weight and feed conversion ratio (Adil et al., 2010). Also, Fructooligosaccharides have been shown to decrease *Salmonella enteritidis'* susceptibility to colonize poultry (Bailey et al., 1991). Enzyme supplementation has been used to improve digestibility in poultry but does not significantly impact the size of the bacterial population in the small intestine (Shakouri et al., 2009).

The alternatives discussed in this review offer promising evidence of potential substitutes for various antibiotics uses. However, a particular supplement category under study is the probiotics, which have proven to be a suitable treatment method favorably impacting health, disease prevention, and performance (Deng et al., 2012). The FAO/WHO (2002) defines a probiotic as “live microorganisms that confer a health benefit to the host when administered in adequate amounts.” These live non-pathogenic organisms are administered to individuals to improve GI balance and homeostasis. They colonize the GI tract and compete with pathogenic bacteria, binding to sites in the epithelial wall and providing a balance of beneficial and commensal bacteria (Pineiro and Stanton, 2007).

The potential benefits of probiotic supplementation are documented (Bailey et al., 1991; Desbonnet et al., 2010; Abdel-Azeem, 2013). Potential effects include preventing pathogenic bacteria proliferation, improving digestibility, immunity, and positive effects on behavior. Several studies have illustrated the benefits of probiotics on the integrity and morphology of the small intestine (Gunal et al., 2006; Adil et al., 2010; Beski and Al-Sardary, 2015). Probiotics have been shown to help stabilize the mucosal barrier in the gut (Castillo et al., 2012). Additionally, prebiotics and probiotics increased carcass yield in broilers and reduced mortality compared to controls (Pelícia et al., 2004). These findings suggest that probiotics are a good candidate as a dietary supplement for animals used in production systems as they improve GI health and integrity, affecting productivity through improvements in metabolism.

The study of animal behavior as a welfare assessment

The Five Freedoms of Welfare are a practical and comprehensive checklist to assess the strengths and weaknesses of husbandry systems. One of these Freedoms is the expression of normal behaviors, and it has proven to be an efficient and applicable way to identify changes in the animals that could indicate management issues. The flexible quality of behavioral adaptation is a cost-efficient evolutionary trait, where less energy expenditure is used compared to physiological shifts (Tierney, 1986). Studying an animal's capacity to adapt to changes in the environment, including behavioral adaptations, offers insight into the animal's overall state (Veisser and Boissy, 2007). In the poultry industry, producers focus on changes in behavior and the expression of maladaptive or abnormal behaviors of the flock (Bracke and Hopster, 2006).

The domestication of poultry dates back to more than 7000 years from their ancestor, the Red Jungle Fowl (West and Zhou, 1988). Behavioral repertoires expressed by poultry species have remained comparably similar to their ancestors (Appleby et al., 2004). Although domestication does not usually add new behaviors, it does change behavioral displays and reallocation of energy expenditure. By providing animals with food, shelter, and protection from predators, they can invest that energy in production traits compared to breeds not selected for high production demand (Schütz and Jensen, 2001).

Conventional housing conditions and management practices stem from the high demand for egg products but have the potential to cause maladaptive behaviors in the hens (Dawkins et al., 2004). These conventional systems consist of metal cages that commonly hold 5-6 hens per cage. High stock densities limit the bird's movement and stifle their capacity to perform natural behaviors (Shimmura et al., 2007). Therefore, alternative housing systems were developed, including enriched cages, cage-free, free range and aviary systems (Shini, 2003; Tauson, 2005; Sherwin et al., 2010). However, stress-related behavior, like aggression and fearfulness, persisted throughout the new housing conditions applied, proving the focus of housing conditions to be limited in its ability to address all aspects of hen well-being (Appleby et al., 1991; Rushen, 2003).

Distinguishing between a bird’s social, sexual, survival, and comfort behaviors can positively impact the management practice by providing clear identification of potential issues (Mauldin, 1992). Pecking order is a ubiquitous behavior in chickens through which they establish a social hierarchy between dominant and subordinate members of the flock. Nonetheless, comb size and body size can establish hierarchy without encountering aggression (D’Eath and Keeling, 2003). This hierarchal organization has evolutionary implications in feeding access, mate choice, and space reallocation (McBride and Foenander, 1962). Craig and Guhl (1969) noted a territorial dominance associated with pullets spending time in a particular area of the pen in larger flocks. The pecking order of dominant pullets usually assigns this territorial dominance. Thus, social rank reduces aggression between flock members by maintaining balanced interactions assigned by the pecking order (Guhl, 1968). In large groups, commonly seen in broiler systems and cage free layer systems, chickens constantly encounter strangers, which hinders their ability for social discrimination and, consequently, the establishment of hierarchy (Estevez et al, 1997).

Social behavior can be any interaction an individual has with their counterparts. Some of these are parenting, allelomimetic, competitive, and courting behavior. Social learning is a predominant trait in chickens, as they mimic their parents and other members of their flock (Hughes, 1998). Chickens are precocial at hatch and are exposed to conspecifics. In mixed-age flocks, they can learn from older birds until reaching maturity (Lindqvist et al., 2007). However, in commercial settings, birds are housed with conspecifics of their same age, so they do not have the opportunity to learn from older birds.

The fifth Freedom of Welfare states promoting natural behavior is an effective method of achieving positive welfare in a commercial setting (FAWC, 1992). Nonetheless, defining what constitutes “normal or natural” behavior complex. Also, a balance between what we call “negative” and “positive” behaviors indicates the animal trying to achieve homeostasis when presented with a changing environment. Additionally, adequate space allocation for feeders can provide more space for locomotor activity in birds, potentially reducing aggression due to competition for food (Thogerson et al., 2009). However, distinguishing the cause of these stressors is complicated and multifactorial (Lay et al., 2011). The expression of behavior outcomes is often species-specific and varies depending on the exposure to the environment and other species (Mench, 1992). Chickens are both prey and predatory that undergo physiological adaptations to fearful encounters. Therefore, the expression of stress-related behaviors is an essential physiological measure of welfare (Appleby et al., 2004).

Fear-related behavior in poultry

In an animal setting, a suitable definition of stress is "a biological or physiological response from an individual following an unknown stimulus that can pose a threat to its homeostasis" (Moberg and Mench, 2000). This definition provides a framework to differentiate the escalation from stress to distress and how these chronic stressors consociate with fear (Moberg and Mench, 2000). When animals are exposed to stressors, they create resistance and adaptations to the changes in their environment and host defense to diseases. Induced stress in birds, caused by normal behavioral inhibition, can decrease egg production and increase cost of egg production as well as flock mortality and morbidity.

Exposure to persistent stressors can result in agonistic behaviors such as aggressive and feather pecking, avoidance behavior, and cannibalism. When a threat is perceived, subordinate birds practice different avoidance behavior modes by staying immobile or trying to escape. They also exhibit neurophysiological changes, affecting performance and mortality (Appleby et al., 2004). Although this energy expenditure can be beneficial in the wild, repeated expression of agonistic behaviors can negatively impact animal welfare (Appleby et al., 2004).

High stocking densities can result in increased feather pecking, negatively affecting meat quality and overall health (Bilčik and Keeling, 2000). Foraging and pecking are part of a bird's natural behavior. Nonetheless, redirected pecking is the most common and severe display of damaging pecking to conspecifics (Shea et al., 1991). A lack of foraging material and the substitution for pelleted food causes an increase in feather pecking, most likely due to redirection of pecking (Aerni et al., 2000). Beak trimming is a common practice in industry in the US to reduce cannibalism and other aggression-related interactions in the flock. However, the chicks suffer acute pain after trimming due to the hundreds of nerve endings they have in their beaks (Benson and Rollin, 2004). On the other hand, non-trimmed flocks have increased injury and cannibalism due to damage from severe feather pecking and aggressive interaction. Therefore, the expression of maladaptive behavior, like feather pecking, can be motivated by many factors.

Stress and the Neuroendocrine system

Biochemical mechanisms in the organism's brain activate the hypothalamic-pituitary-adrenal (HPA) axis, regulating behavioral adaptations after exposure to stressful stimuli (Stephens and Wand, 2012). Subsequently, this activation results in a change in neurotransmission: chemical substances responsible for transferring information between neurons in the body (Cheng et al., 2001). Many of these neurotransmitters, such as serotonin (5-HT), dopamine (DA), epinephrine (EP), and norepinephrine (NE), are involved in the regulation of aggressive and antisocial behaviors (Haden and Scarpa, 2007; Cheng et al., 2001). Both external and internal stressors can elicit a HPA response in poultry, and the release of neuromodulators, neurotransmitters and hormones causes a change in their behavioral repertoire (Lay et al., 2011).

The gut microbiome has a role in peptide and nutrient breakdown, and some of the metabolites are involved in neural function (Stilling et al., 2013). The bidirectional communication between the brain and the gut is known as the gut-brain axis (GBA). Several hormones are released to the central nervous system (CNS), affecting GBA functioning (O' Mahony et al., 2011). In hens genetically selected for high and low group survivability, the hens with the lowest survivability also had higher blood concentrations of DA, EP: NE, and 5-HT. (Cheng et al., 2001). The relationship between neuroendocrine homeostasis, coping strategies, and changes in behavioral patterns is delineated. Differences in chickens' genetic coping adaptations have been associated with different neuroendocrine hormone levels (Cheng and Muir, 2007). At a physiological level, neurotransmitters interact with other systems and organs for their expression. The gastrointestinal microflora of chickens is still not fully understood. The sections with the most bacterial activity in the GI tract of chickens are the crop and the caeca, and includes lactobacilli, enterococci, and enterobacteria. Determining the relationship between the enteric nervous sytem (ENS), brain function, and GI interactions will serve as a tool to study animal behavior in intensive systems.

Probiotic supplementation may affect gut microbiome processes (Yildirum et al., 2020). Probiotic supplementation in turkey reduced behavior associated with distress and aggression (Abdel-Azeem, 2013). Broiler chickens supplemented with *Bacillus* had coping mechanisms to modulate behaviors associated with heat stress without affecting production performance (Wang et al., 2018). A similar pattern was observed with *Bacillus* supplementation in laying hens, where its improved egg production and intestinal integrity and other performance qualities that are commonly disrupted when under heat stress (Deng et al., 2012). Understanding what internal processes are occurring for probiotics to improve or change behavior will prove helpful to eventually apply this to the behavioral problems often seen in production systems.

Serotonin's impact on physiology and behavior through Tryptophan

An animal's reaction to external stimuli corresponds with internal mechanisms that are crucial when studying behavioral neurophysiology. Specifically, how hormone secretion and availability come into play when expressing specific behaviors. In animal models, 5-HT, DA, CORT, and NE, acting as both neuromodulators and hormones, have provided a framework of the internal stress response messages sent through the body (Cheng et al., 2001). For behavioral phenotypes in poultry, 5-HT is extensively studied due to its sedative-like effect that could be promising when dealing with agonistic behaviors in production systems. Serotonergic systems are involved in many regulating processes such as mood, arousal, aggression, and feeding behaviors (Olivier et al., 1998; Young and Leyton, 2002; Martin et al., 2012). The effect of 5-HT occurs mainly through tryptophan (Trp) administration. In multiple animal models, aggression is potentially inhibited through receptor pathways, 5-HT1A/1B (de Boer and Koolhaas, 2005; Dennis et al., 2008), but exact mechanisms are not fully elucidated. Therefore, the 5-HT precursor, Trp, influences behavior changes, neuroendocrine processes, and overall performances.

When referring to 5-HT's ability to impact behavior, it is imperative to discuss the influence and importance of its precursor, Trp. Tryptophan is responsible for protein synthesis, biological metabolites, and other critical physiological processes (Le Floc'h, 2011). This essential amino acid is administered to animals through the feed as it is not synthesized in their bodies. Interestingly, only around 10% of the total Trp breakdown is utilized to produce 5-HT, most of it turning into kynurenic acid and quinolinic acid through the kynurenic pathway (Höglund et al., 2019). To produce 5-HT, Trp is hydroxylated to 5-hydroxytryptophan by the enzyme Trp hydroxylase (TPH, rate-limiting enzyme) and decarboxylated to 5-HT by the aromatic amino acid decarboxylase (Höglund et al., 2019). Ninety-five percent of this peripheral 5-HT is produced in the GI tract, where serotonergic cells extend throughout, mediating motor and sensory functions. (Tecott, 2007). It is synthesized and stored in enterochromaffin (EC) cells that reside within the intestine wall's epithelium and are modulated by the enteric nervous system.

Depletion of Trp affects behavior expression and stress response (Russo et al., 2009). In mice, administration of 5-HT agonist after isolation had an anti-aggressive effect. (Gibbons et al., 1979). Prenatal administration of 5-HT can result in changes in chicken's postnatal behavioral phenotype (Dennis et al., 2013). During early development, aggressive poultry strains administered with 5-MT, a 5-HT agonist, corresponded with a higher fear response in a dose-dependent manner (Dennis et al., 2013). A similar pattern was observed with prairie voles when administered 5-MT (Martin et al., 2006).

Given the impact that 5-HT has in mediating aggression, supplementary Trp could serve as a tool to address these behaviors. As mentioned earlier, chickens in intensive production systems may display maladaptive behaviors, whether fearful or aggressive. In chickens, coping mechanisms are related to changes in the internal homeostasis or expression of hormones. White Leghorn lines with a high incidence of feather pecking (FP) had higher peripheral levels of 5-HT than chickens with a lower FP incidence (Buitenhuis et al., 2006). Given that Trp is a precursor of 5-HT, depleting the animal's diet of basal Trp could result in the onset of negative behaviors due to its effect on 5-HT activity. Depletion of both could have adverse behavioral consequences like aggression or other compulsive behaviors (Fineberg et al., 2010). The mechanism by which peripheral 5-HT affects behavior is unclear but relies, in part of having sufficient levels of available Trp.

Feed restriction is used extensively in poultry production, but it usually is followed by higher levels of aggression through dominant broiler breeder males. Supplemental dietary Trp significantly reduced aggression in skip-day (SD)-fed broiler breeder males once the peck-order was established (Shea and Thomas, 1990). In laying hens expressing panic-like behaviors, dietary Trp increased the hypothalamic serotonin levels and decreased episodes of hysteria (Laycock and Ball, 1990). As a feed intake enhancer, studies show that Trp deficient diets in broiler resulted in low body weight (BW), feed intake, and feed conversion (FCR) (Corzo et al., 2005). Aggression decreased in dominant male birds through Trp's actions (Shea et al., 1991).

Gut microbiome physiology and gut-brain-axis interactions

The GBA forms essential physiological roles such as nutrient breakdown, peptide function, and immune response. The by-products broken down by the gut microbiota are absorbed and utilized at the enteric site of the peripheral nervous system, influencing neural function (Stilling et al., 2013). Studies in mice revealed the capacity of the gut microbiota to express biologically active catecholamines in the lumen of the gut (Asano et al., 2012). Therefore, the homeostasis of the GI tract's microflora exerts a significant influence on brain function and the production of neurochemicals as a basis for behavior.

There are physiological and morphological outcomes of homeostatic disruption in the intestinal tract. Exposure to stressors causes a decrease in villi length in quails (Konaka et al., 1979; Deng et al., 2012). Villi are the modes of nutrient absorption in the GI tract, so their morphological decrease results in a poor metabolism through an impairment in feed efficiency, reduced feed, and water intake, and reduced body weight. Villi length is crucial for metabolic processes, and in laying hens it has been found that a decrease in villi length results in inflammation of the small intestine (Deng et al., 2012).

In a study by Park et al. (2013), the resident microbes in the GI tract were significantly altered following the induced depression in mice. Gram-negative and gram-positive bacteria translocated to the liver after being in these stressful environments, meaning the release of certain hormones upon exposure could deteriorate the permeability of internal organs and bring translocation of microbiota (Bailey et al., 2006). The morphological integrity of the GI tract is disrupted following exposure to heat stress, resulting in bacterial translocation (Burkholder et al., 2008). Results from Bulkhorder et al.'s (2008) study showed that the similarity of commensal bacterial populations in the ileum and the number of species in the ileal wall decreased upon exposure to heat stress. Disease susceptibility can happen when there is a disturbance in the balance of commensal and non-commensal bacteria and frequent problems with gastrointestinal inflammation.

Inflammation in the small intestine is a cause of disease in both humans and non-human animals, but it also has a strong influence on behavioral changes (Bercik et al., 2010). Infection with *Citrobacter rodentium* at an early stage in mice resulted in anxiety-like and fearful behaviors compared to their healthy counterparts (Lyte et al., 2006). In murine models, social disruption caused by a male aggressor is reflected in a loss of microbiota diversity (Bailey et al., 2011). Research conducted with GF mice depicts similar results. GF mice expressed more exploratory behavior compared with SPF mice with normal microbiota, while at the same time expressing higher concentrations of CORT, norepinephrine (NE), DA, and 5-HT, hormones known to affect behavior (Neufeld et al., 2010; Heitz et al., 2011). Therefore, an interplay exists between the gut microbiota homeostasis and behavior affecting the animal’s performance and its well-being.

For a bacterial strain to be effectively used as a supplemental probiotic, first it must be a genetically stable and non-pathogenic microorganism. Second, once it reaches the aggressive GI tract, it must survive, metabolize and adhere to the GI tract walls, Third, the bacteria should modulate immune response while having proven benefits to the host (Hajati and Rezaei, 2010). Although the GI tract has its functionality in protecting itself, an increase of potential stressors, infection or malnutrition could prevent it from performing it efficiently. As probiotics are naturally occurring bacteria that are usually present in the gut, supplementation can positively affect GI function. Probiotic supplementation can positively impact bodyweight, daily gain, and immunity in a similar way to antibiotics while reducing pathogenic bacteria and promoting beneficial microbial (Teo and Tan, 2007; Yang et al., 2012).

Microorganisms and the expression of tryptophan in the GI tract

Microorganisms are involved in Trp degradation through the actions of various enzymes (Gummalla and Broadbent, 1999). Under stressful conditions, the supplementation of different strains of *Lactobacillus* reduces circulating levels of CORT and ACTH (Bravo et al., 2011; Ait-Belgnaoui et al., 2012). *Lactobacillus* added to basal diets increased body weight in broilers (Jin et al., 2000). *Lactobacillus* is 5-HT producing probiotics. Strains of *Lactobacillus* have reduced depressive-like behavior in mice while ameliorating 5-HT levels. Studies show how *Lactobacillus* can promote colonic 5-HT synthesis to, in turn, contribute potentially to the improvement in GI motor function and availability of Trp (Hara et al., 2018). In addition to 5-HT production, bacterial response likely plays an essential role in determining local GI and circulating Trp availability for the host, and consequently it affects their host's behavior or metabolism to some degree.

Conclusion

Promoting positive animal welfare is a multifactorial process that involves flock and individual behavior. Through the study of animal behavior, animal welfarists can focus on promoting positive affective states to improve animal welfare. Given that GBA activity influences behavioral expression and physiological processes, changes to this system can alter the way animals react to changes in their environment. Through 5-HT expression, Trp administration can have a positive effect on behavioral expression and physiological processes. Additionally, probiotic supplementation can impact the way Trp reacts at the level of the gut microbiome to cause an effect on the animal's internal processes. Our research aims to understand how Trp, and probiotics can work together to optimize well-being while maintaining high levels of production.

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Chapter 2: Effect of tryptophan administration with probiotics or antibiotics on behavioral patterns in White Leghorn chickens

Abstract

An animal's behavioral pattern can offer insight into their current state, with long-term repercussions to overall well-being. Maladaptive behaviors in poultry production systems have negative implications for animal productivity and welfare. Fostering positive affective states can reduce these negative repercussions. Dietary supplementation can address issues that directly or indirectly affect behavioral expression. We investigated the effects of either probiotic or antibiotic supplementation mixed with tryptophan (Trp) on overall behavior. Female White Leghorn birds were assigned to 6 different treatments in a 2x3 factorial design: Trp-antibiotic (Tryptophan supplementation: 0.32%, and antibiotic treatment: 125mg/L), Control-probiotic (probiotic treatment: 10^9 CFU/L, and basal Trp: 0.16%), Trp-control (Trp supplementation and no additional treatment), Control-antibiotic (antibiotic treatment and basal Trp), Trp-probiotic (Trp supplementation with probiotic treatment), or control (n=12). The Trp-probiotic group exhibited higher frequency of eating and walking ($P = 0.04$ and $P = 0.02$, respectively), and lower frequency of sitting ($P = 0.01$) in comparison with Trp-control ($P > 0.05$). Trp-control group had higher frequency of standing and gentle feather pecking ($P = 0.02$ and $P = 0.04$, respectively) in comparison with control ($P > 0.05$). Results show how variable probiotic and Trp interactions can impact behavior, particularly because excessive

Trp increases bacterial metabolism in the GI tract, competing with probiotic supplementation and their potential effects on positive behavioral expression.

Introduction

Tryptophan (Trp) is an essential amino acid responsible for several physiological processes and changes in behavior. Trp is also an obligatory substrate for the hormones: ghrelin, serotonin, and melatonin (Zhang et al., 2007; LeFloc'h et al., 2011). Serotonin (5-HT) is a biogenic amine that functions as a neurotransmitter, a regulator of gastrointestinal processes (secretion, motility, and sensation), cognition, mood, and appetite, and Trp mechanisms correspond with the possible expressions of such processes. This amino acid has also become of interest for animal welfare research (van de Eijk et al., 2019; Neto and Giaquinto, 2020). Research shows Trp's ability to reduce the onset of aggression by promoting a positive or sedative-like effect (Gibbons et al., 1979; Shea-Moore et al., 1991; Moneva et al., 2008). Therefore, Trp can influence behavioral expression through increased levels of 5-HT.

Changes in Trp availability can alter 5-HT production, ultimately impacting animal behavior and well-being. The serotonergic system's influence on mood and behavior is well documented (Buitenhuis et al., 2006; Tecott, 2007; Falibene et al., 2012). Serotonin is a vital therapeutic in human studies to target anxiety, depression, and other mood disorders (Tecott, 2007). The same sedative-like effect of 5-HT has made it a good hormone in the study of animal welfare in production systems. Often, maladaptive behaviors emerge in farms, and addressing them proves to be challenging in an intensive and dynamic setting. Specifically, aggression and fearful behaviors in chicken production could have detrimental effects on yield. Severe feather pecking is detrimental to the animal's well-being and can cause economic problems to the industry (Huber-Eicher and Sebö, 2001). It is essential to prioritize reducing fearful or antagonist behaviors to optimize well-being in production systems. As mentioned, agonistic behaviors in poultry production negatively affect quality of life. However, other behaviors such as reduced feed intake, excessive sitting, or resting can also indicate some discomfort of the animal (Appleby et al., 2004). Additionally, laying hens have higher social interactions than, for example, broiler chickens. Broiler chickens do not often develop a pecking order as they interact with ever-changing conspecifics.

Approximately 231.7 million laying hens are reared in conventional systems made up of battery cages in the U.S.A. (United Egg Producers, 2021). This housing style stifles social behaviors and negatively impacts their well-being, whether it is developing a pecking order and having subordinate birds eat or drink less or a tendency to be apart or inactive due to fear (Campler and Jensen, 2009). Eating behavior is perhaps one of the most exhibited behavior amongst laying hens, especially during the active laying season (Hocking et al., 1997). Therefore, the prevalence of this behavior hints towards the physiological comfort and balance of the bird. Some of the comfort behaviors observed in poultry are dustbathing, preening, leg and wing stretching (El-Iraqui et al., 2013). The biological resources allocated to express this behavior could indicate that they have satisfied their basic needs and can allocate time to express those behaviors. Focusing on adaptive and maladaptive behavior gives us more accurate data regarding the animal's current state since welfare should also enhance positive behaviors.

Behavioral patterns indicate a constant influx of mood and behavioral changes accompanied by environmental and social outcomes. Elevated levels of Trp could potentially ameliorate the negative behaviors often seen in poultry production or enhance positive ones. Given that around 95% of 5-HT is produced in the GI tract (O' Mahony et al, 2015), the microbiome has a potential to greatly influence the animal's behavior-regulating systems. The GI tract is composed of bacteria, archaea, and fungi responsible for producing essential metabolites, some of these metabolic products affect behaviors (Cresci and Bawden, 2015). Anxiety-like behavior in mice was induced when infected with *C. jejuni* (Lyte et al., 2021). Impaired expression of natural behaviors in Beijing You chickens correlated with changes in their microbial diversity (Chen et al., 2019). Lastly, probiotic supplementation altered the behavior of Ross Broilers following a stress challenge (Rehan et al., 2020). The cascade of mechanisms involved in the expression of such behaviors could be impacted by the microflora present in the gut.

Gut microbiome interactions can influence behavior when additional microorganisms are added, aiding in the metabolic processes responsible for producing hormones that affect behavior. The use of probiotics as dietary supplements has resulted in solving problems associated with production like heat stress (Zulkifli et al., 2000). In some cases, antibiotics can inhibit essential processes carried out by the gut microbiome due to a depletion of beneficial bacteria, thus disrupting homeostasis (Iizumi et al., 2017). It is not clear to what degree this occurs in poultry production, particularly in laying hens due to their limited antibiotic administration. These supplementation techniques have become popular in targeting welfare issues because they are less intrusive and applicable to farming scenarios. (Cahir et al., 2007).

Alterations in the amount of available Trp causes changes in mood and coping styles (Young and Leyton, 2002). This study aimed to better understand the possible biochemical interactions between the 5-HT precursor, Trp and microbiota altering supplements, probiotics and antibiotics. We further examined how this interaction can alter behavioral states. Our study focused on altering behavioral phenotypes through supplementing laying hens with the amino acid Trp and the microbiome altering supplements probiotics and antibiotic, in a factorial design.

Methods

All procedures involving live birds were approved by University of Maryland Institutional Animal Care and Use Committee before initiation of the experiment (#981251-1).

One hundred and forty-four female White Leghorn chickens (*Gallus gallus)* at 16 weeks of age were used for this study. Birds were marked with green or blue livestock markers and placed in wired layer cages hosting two birds per cage at the Animal facility building at the University of Maryland. The same protocol was applied to all birds for consistency in dietary and environmental background. A standard layer diet was administered until hens reached 90% lay (21 weeks of age). Food and water were given *ad libitum.* Thereafter, one of five treatment diets or control diets was assigned. Health checks, feeding, and egg collection were done daily.

Dietary treatments

Tryptophan was part of the basal diet at 0.16% (control). Additional Trp supplementation at 0.32% was added to the diet for the other treatments (to a total of 0.48% dietary Trp). Microbiome altering treatments consisted of a probiotic mix (*L. acidophilus, B. bifidum, B. breve, B. infantis, B. longum, L. fermentum, L. casei, L. rhamnosus, L. plantarum*; 10^9 CFU/L or antibiotic administration (Erythromycin; 125 mg/L) both were added to the water (Table 1; 12 cages/treatment; 2 birds/cage). The cages were evenly assigned six treatments on a 2x3 factorial design and assigned to cage by randomized block design: 1) Control-control (control with no Trp supplementation); 2) Trp-antibiotic (Trp supplementation and antibiotic treatment); 3) Control-probiotic (no additional Trp with probiotic treatment); 4) Trp-control (Trp supplementation with no additional treatment); 5) Control-antibiotic (no additional Trp with antibiotic treatment); and 6) Trp-probiotic (Trp supplementation with probiotic treatment). Both probiotic mix and antibiotics were added to the water, which was changed daily. Following 10 days of dietary treatment, hens were transferred to new home cages. Cages were of the same dimensions as the previous home cages, with a novel cage mate assigned. Since new cage mates observed were novel to each other and received their treatment independently, the bird was considered the experimental unit for behavioral analysis.

Table 1. Dietary components of Trp, probiotic, or antibiotics treatments

Diet Treatment	**Trp supplement**	**Trp total**	**Probiotic supplement**

Control-control	0%	0.16%	No supplement
Control-antibiotic	0%	0.16%	Antibiotic
Control-probiotic	0%	0.16%	Probiotic mix
Trp-control	0.32%	0.48%	No supplement
Trp-antibiotic	0.32%	0.48%	Antibiotic
Trp-probiotic	0.32%	0.48%	Probiotic mix

In cage focal behavioral tests

For behavioral analysis, focal animal sampling was performed on novel cages. All cages were observed via 700TVL analog cameras and Zmodo multi-channel security system DVR recorders. The observational methods were focal behavioral tests, where each bird was observed continuously for 30 minutes. Behaviors were recorded for 30 minutes immediately following the introduction into the new cage and for 30 minutes after being in the cage for 8 hours. A graduate student, two undergraduate students, and one professor analyzed behaviors using JWatcher software. For analysis, behavior was divided into normal/natural (stand, sit, walk, drink, eat), comfort (preen, wing flap), and peck (peck cage, gentle feather pecking) behavior (see Appendix I for ethogram).

Statistical analysis

Analysis of the main effects and interaction of the tryptophan and probiotic treatments were done using two-way ANOVA method. All data were tested for normality of distribution and variance. Log transformation was utilized when appropriate. Means of the data were analyzed by using a mixed model with SAS 9.4 software (SAS, Cary, NC) as a factorial arrangement of 2 x 3. Fixed effects in the model included main effects of tryptophan and probiotic and the interaction on behavior. For ANOVA, P-values less than or equal to 0.05 were accepted as significantly different. The sample sizes for this result section (n= 139), results shown in LS means + SEM, F-value, and p-value.

Results

Natural behaviors

There were significant interactions between the Trp and microbiome altering treatments on sitting, standing, walking, eating and drinking behaviors (Figures 1-5). The Trp-probiotic group spent significantly less time sitting (0.41 ± 0.23) compared to Trp-control and Trp-antibiotic group (0.55 ± 0.23 and 1.46 ± 0.23, respectively; $F_{5, 138}$= 4.42, P = 0.01; Figure 1). The frequency of standing behavior was significantly higher in the control-probiotic group (3.58 ± 0.1) than control-control and control-antibiotics (3.12 ± 0.1 and 3.27 ± 0.1, respectively; $F_{5, 138}$ = 2.39, P = 0.02; Figure 2). Walking behavior was increased in Trp-probiotic (10.70 ± 1.32) in comparison with Trp-control and Trp-antibiotic (6.02 ± 1.32 and 6.15 ± 1.32, respectively; $F_{5, 138}$ = 2.50, P = 0.02; Figure 3). Trp-antibiotic significantly decreased eating (2.86 ± 0.2) when compared with Trp-probiotic (3.3090 ± 0.19; $F_{5, 138}$ = 2.02, P = 0.04; Figure 4). Control-control exhibited drinking behavior significantly less (0.4751 ± 0.20) than other treatments (Control-antibiotic 1.3043 ± 0.20, Control-probiotic: 0.9864 ± 0.20, Trp-control: 0.9559 ± 0.20, Trp-antibiotic:1.1068 ± 0.20, Trp-probiotic: 1.1840 ± 0.20; $F_{5,\,138} = 2.60$, $P = 0.01$; Figure 5).

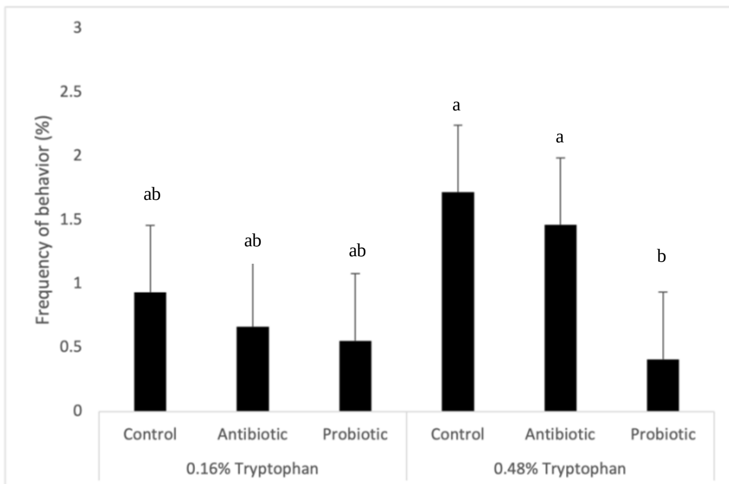

Figure 1. Average frequency of sitting by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments (p <0.05).

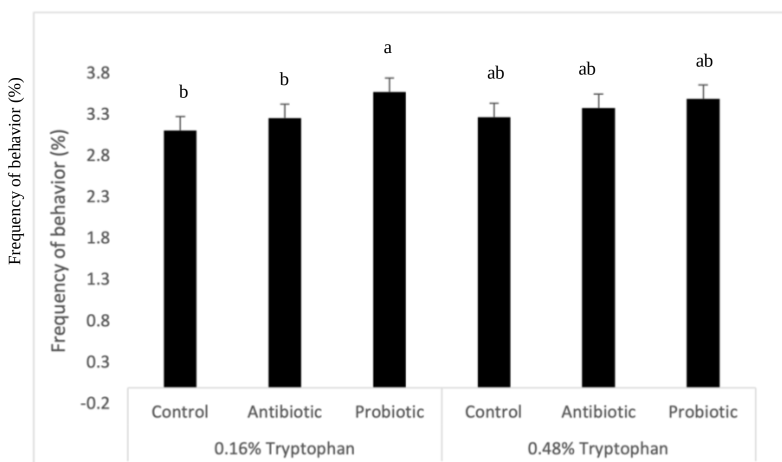

Figure 2. Average frequency of standing by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments (p <0.05).

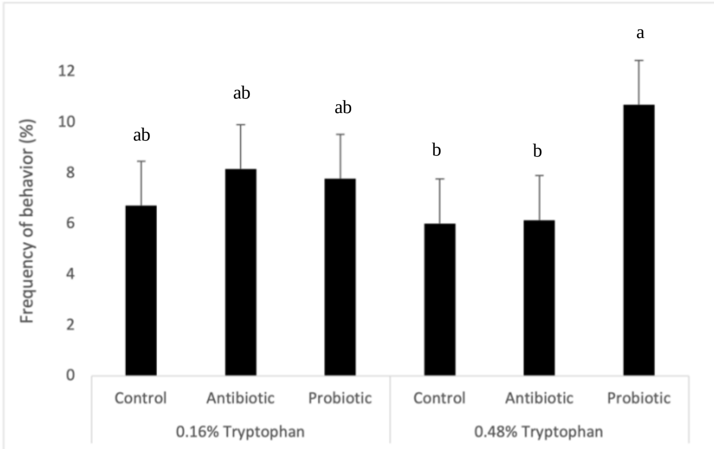

Figure 3. Average frequency of walking by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments (p <0.05).

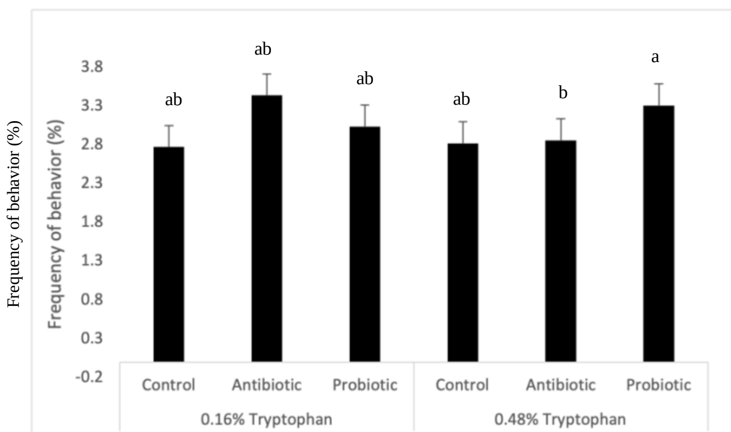

Figure 4. Average frequency of eating by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments (p <0.05).

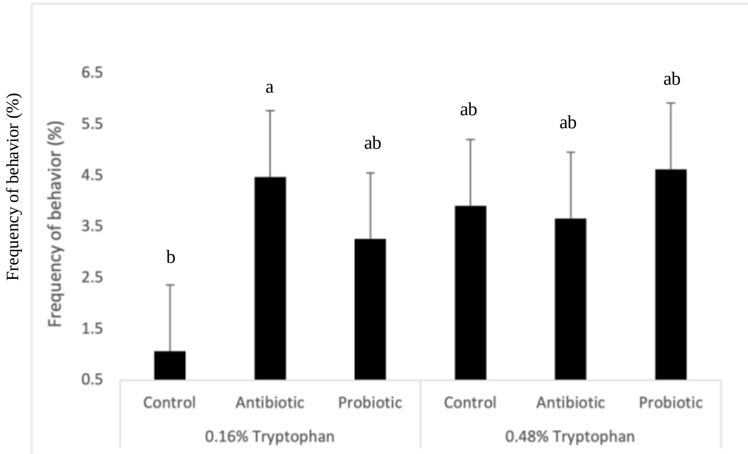

Figure 5. Average frequency of drinking by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments (p <0.05).

Comfort behaviors

Control-control birds spent significantly more time preening (6.18 ± 1.78) than control-probiotics (1.19 ± 1.78; $F_{5, 138}$ = 3.47, P = 0.04; Figure 6). Wing flapping was not affected by any of the treatments ($F_{5, 138}$= 3= 0.67, P = 0.65; Figure 7).

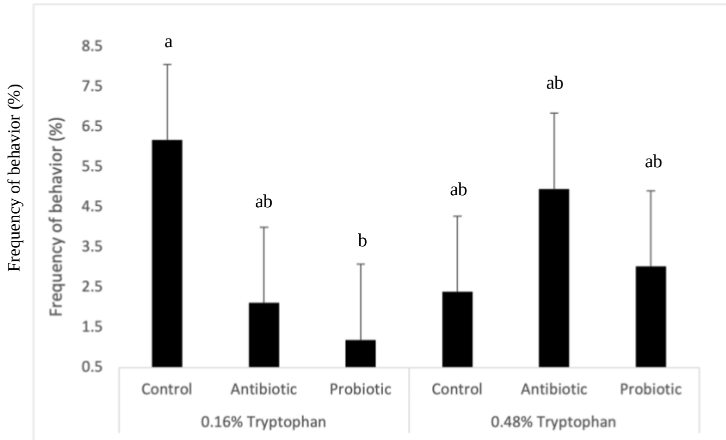

Figure 6. Average frequency of preening by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments (p <0.05).

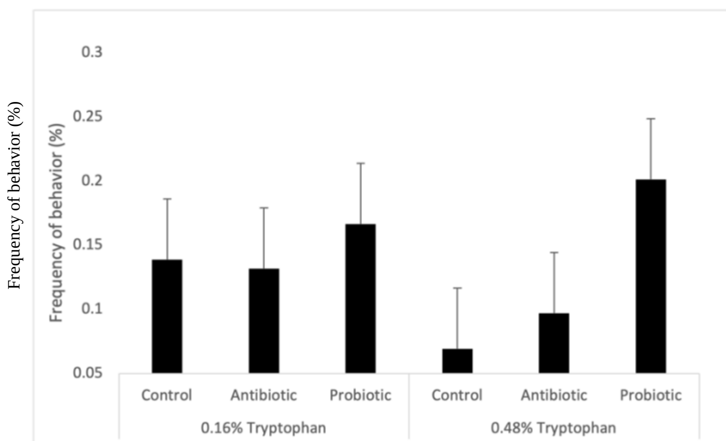

Figure 7. Average frequency of wing-flapping by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments (p <0.05).

Pecking behaviors

Control-antibiotics birds pecked the cage the most (0.64 ± 0.10) in comparison with control-probiotics (0.36± 0.10; $F_{5, 138}$ =2.25 P = 0.03; Figure 8). Trp-probiotic group (1.35 ± 0.63) gently feather pecked more than the Trp-antibiotics birds (0.83 ± 0.63; $F_{5, 138}$ = 2.04, P = 0.04; Figure 9).

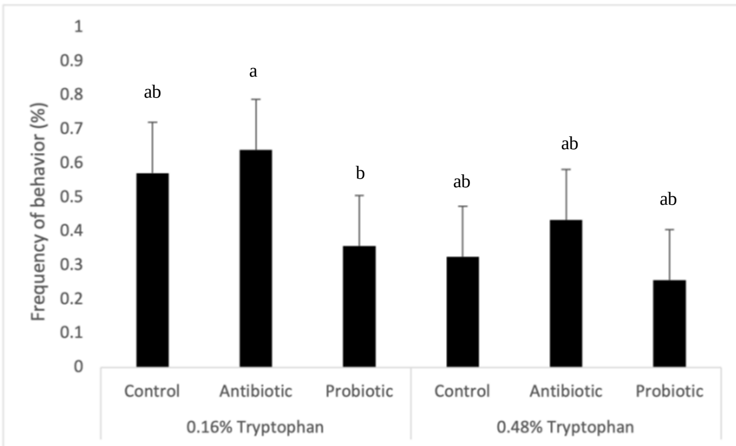

Figure 8. Average frequency of cage pecking by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments ($p < 0.05$).

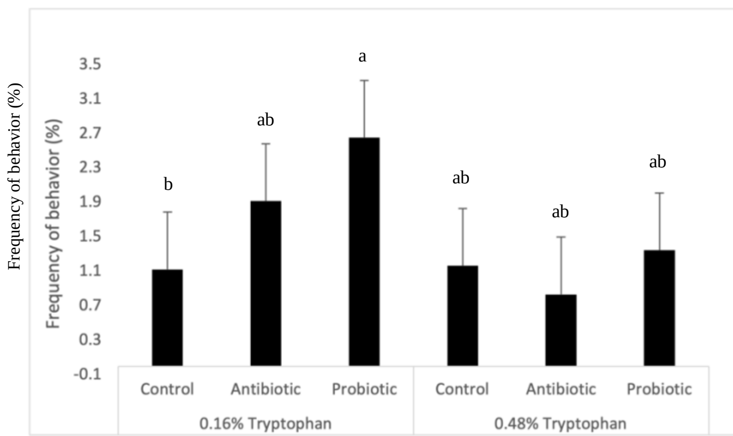

Figure 9. Average frequency of gentle feather pecking by birds after Trp and probiotic/antibiotic treatment. Bars represent means ± SEM. Different letters above bars indicate significant differences between treatments ($p < 0.05$).

Discussion

Our results show that dietary supplementation of tryptophan and probiotics can impact normal, comfort and pecking behaviors. Previous studies on the supplementation of additional Trp have met with varied results, depending on digestibility, competition with other amino acids, and nutrient absorption (Yildirim et al., 2020). Similarly, our data suggest that the effects of dietary Trp on behavior can be altered by microbiome altering treatments.

Birds supplemented with additional Trp and probiotics walked significantly more while also reducing their sitting frequency in comparison with Trp alone and Trp and antibiotics groups. The ability to move unrestricted and perform natural behaviors is one of the freedoms of welfare stated by FAWC (1992). Although sitting can be associated with a relaxed or comfortable behavior, an excess of sitting behavior can also indicate that the animal is distressed or fearful, as it does not interact with its environment. In contrast, walking behavior was higher, which is positive since we want the animals to exhibit locomotion activity. Previous studies with synbiotic supplementation have a similar increase in activity, suggesting improvements to the bird's musculoskeletal health (Mohammed et al., 2018). Supplementing Bacillus to Japanese Quail improved their musculoskeletal strength (Alam et al., 2020). Our results demonstrate that Trp and probiotics interactions in the GI tract allowed the animals to perform higher locomotion activity.

Eating behavior was higher in birds supplemented additional Trp with a probiotic, when compared to those supplemented with Erythromycin. Serotonin is involved in the regulation of appetite (Capello et al., 2014), and disturbances in the GI tract can affect this control system. As *Lactobacillu*s is a 5-HT producing probiotic and was one of the strains used in our study, it may hint towards how that can be associated with increased feeding. On the other hand, a reduction of feeding behavior can reduce hen weight and egg production. Having dietary alternatives that could motivate an increase in eating helps assure that most of the animals in the flock are getting their adequate energy requirements through feed, thus reducing any suffering associated with lack of eating.

An animal's expression of comfort behaviors depends on its ability to reallocate energy for preening, wing flapping and other such behavioral displays. In our studies, preening behavior was higher in control groups, and no significant differences were observed in wing-flapping behavior. Comfort behaviors are an adaptive coping mechanism (Delius, 1988). The manipulation of the feathers is an example of desirable grooming behavior. This behavior also depends significantly on the housing conditions where birds are reared. Wing flapping can be challenging in a small enclosure but preening of some degree can be performed with less difficulty.

Pecking at the cage was decreased in hens fed additional Trp compared to controls. A high incidence of pecking cage behavior can become a stereotypical behavior, indicative of poor welfare. In contrast, gentle feather pecking was expressed at a higher frequency in birds supplemented with probiotics. Gentle feather pecking corresponds with positive behaviors expressed by hens when they have the motivation and environment to undergo social grooming with conspecifics. In studies with pigs supplemented with Trp, there was a reduction in hormone levels associated with stress response, but the expression of stress behaviors measured in that study did not change (Koopmans et al., 2005). Expression of behavior depends on multiple factors outside of endocrine response.

The gut-brain axis (GBA) is responsible for the bidirectional communication of the gut microbiome and the brain (Foster et al., 2016). The activities of the GI tract influence the number of neurotransmitters present in its microbiome and, consequently, behavioral changes. Diet composition influences the microbiota activity since nutrients are turned into metabolites by the microorganisms that make up the gut microflora. These metabolites are short-chain fatty acids (SCFA), biogenic amines, monoamines, such as 5-HT (Le Floc'h et al., 2011). Serotonin production depends on the availability of Trp, and this amino acid competes with large neutral amino acids (LNAA) to cross the blood-brain barrier (BBB) (Shen et al., 2012; Sterndale et al., 2020). As Trp reaches its threshold of availability, only less than 5% of the amino acid is used to convert 5-HT. In our study with Trp supplementation, many of these metabolic processes may have influenced amino acid production of 5-HT and, consequently, the expression of behaviors associated with the neurotransmitter.

The intrinsic neurons in our gut, enterochromaffin (EC) cells, are responsible for communicating with the brain and releasing neurotransmitters which interact with gut microbiota (Bullich Villarubias, 2015). Serotonin production comes from the chemical breakdown of anaerobic fermentative bacteria. Through the release of SCFA, they induce the production of 5-HT through EC cells. Specifically, Lactobacillus aid in tryptophan mechanisms, (Bullich Villarubias, 2015), which would indicate potential changes in behavior and physiology of the animal. Given that behavioral and physiological changes affect the animal's well-being, and how the gut-brain-axis is involved in these processes, careful consideration should be given to the gut microbiome.

Gut microbiome processes should be considered in husbandry systems when modifying management and diet. Disturbances to the gut microbiome could cause a cascade of behavioral changes, including increased stress, anxiety, reduced feed intake, or general pain. Maintaining a diverse microbiota improves host health through better digestion, nutrient absorption, and overall comfort. Probiotics improve the gut's health by reducing pathogenic bacteria, establishing beneficial bacteria, improving peristaltic movements, and balancing pH (Naka et al., 2005; Ohimain and Ofongo, 2012). Additionally, Trp catabolites influence the immune system (Friedman, 2018). Although our study showed high variability in the expression of natural behaviors, other ways of indirectly affecting behavior exist. An animal's health also affects behavioral expression. Sick animals can have restricted movement, may not metabolize feed correctly, and stifle natural behavior, all of which negatively affect welfare. Therefore, Trp and probiotic supplementation can indirectly affect behavior through their influence on poultry's health.

Our results offer insight into the interactions between dietary and microbiome altering supplements on behavioral expression in laying hens. Aspects of behavioral changes are multifactorial, often resulting from a cascade of events. In our study, we saw precisely that occurrence, where behaviors were affected by individual supplementation of probiotics and Trp along with interactions between the two. More research is needed relating to gut microbiome analysis on individual birds, and the impact on behavioral displays. Additionally, probiotic supplementation research should remain ongoing to determine the most ideal probiotic to be implemented in poultry production, with a strong basis for positive behavioral expression.

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Chapter 3: Effect of tryptophan administration with probiotics or antibiotics on production performance and egg parameters in White Leghorn chickens

Abstract

A focus on the application of probiotics to improve poultry performance and animal well-being has been underway. Probiotics have shown promising capacities of improving digestibility and gut motility for better production performance and egg production in laying hens. Their interaction with tryptophan (Trp) can also aid in serotonin (5-HT) expression via gut-brain-axis interactions, regulating physiological functions. Thus, this study was conducted to evaluate the effect of probiotic supplementation and Trp, a 5-HT precursor, on production performance and egg production response of White Leghorn layers birds. Female White Leghorn birds were assigned to 6 dietary treatments in a 2 x 3 factorial design: Trp-antibiotic (Tryptophan supplementation: 0.32%, and antibiotic treatment: 125mg/L; n=12), Control-probiotic (probiotic treatment: 109 CFU/L, and no additional Tryptophan: 0.16%; n=12), Trp-control (Trp supplementation and no additional treatment; n=12), Control-antibiotic (antibiotic treatment and no additional Trp; n=12), Trp-probiotic (Trp supplementation with probiotic treatment; n=12), or control (n=12) to observe potential changes regarding production performance (Bodyweight, liver weight, heart weight, fat pad weight) or egg parameters (egg weight, egg production, and daily egg yield). Results showed that the control group had the highest body weight (P = 0.03) compared to all other treatments (P > 0.05). Additionally, the groups supplemented with Trp and Erythromycin had an increased fat pad and heart weight (P = 0.04

and *P* = 0.04, respectively) than Erythromycin alone (*P* > 0.05). No differences were observed in egg parameters in this study. Trp and probiotic supplementation impact production parameters, however, interactions between the two offer variable results. These results suggests that the effects of probiotics are multifactorial and can interact with mechanisms occurring at the level of the gut microbiome, consequently impacting poultry performance.

Introduction

The application of practices that promote positive animal welfare have ramifications for zoos, companion animals, and animal agriculture. However, it can be challenging to apply all parameters necessary for ideal animal welfare in intensive animal production, given that producers face other challenges, such as market demands, adjusting to technological advances, and other management responsibilities. For this reason, it is vital to find inexpensive and effective ways in which husbandry systems can make positive changes.

Poor welfare can also directly or indirectly affect producers. For example, severe feather pecking caused by stressors can ultimately kill their animals or affect the meat quality and production capacity (Bilčik and Keeling, 2000). In addition, reduced body feather cover caused by antagonistic social interactions can reduce the animal's ability to thermoregulate, which can negatively affect the animal's health and increase the cost of production. Moreover, although producers lose animals daily due to the inevitable causes, controlling mortality to some degree will be effective for their monetary gain in the industry.

Amongst the challenges that poultry producers face are the misconceptions among the public surrounding housing conditions, stocking densities, and antibiotic use. It is clear that battery cages have undesirable qualities, but other housing conditions face similar limitations. Alternatives housing like free-range face increased predation and susceptibility to foreign parasites. Although antibiotics are not commonly used in laying hens, the general public has associated their use in broiler chickens with being used in layers. Contrary to broilers, laying hens are not given antibiotics as growth promoters. However, the general public tends to integrate both their issues interchangeably, which creates a lot of misinformation and pressure on the producer.

Although there is more pressure on welfare standards requirements in the E.U., the U.S. is slowly making changes to improve animal well-being – primarily through welfare focused niche markets and not changes to federal regulation. In the U.S.A., more than 70% of laying hens operations are in battery cages (United Egg Producers, 2021). This type of housing is the most effective regarding productivity and disease control (van Horne and Achterbosch, 2008). However, a welfare concern associated with battery cages is that the birds cannot express natural behaviors due to such enclosures' space limitations. Aggression is reduced compared to broiler breeder chickens which have management practices that increase aggressive behaviors, such as skipping a feeding day (Shea et al., 1991; Girard et al., 2017). Dominant or aggressive behavior in birds reared in conventional systems can impact productivity due to territorial dominance on feed troughs, making it inaccessible for subordinate animals to access food without receiving aggressive pecks (Craig and Guhl, 1969).

Dominant-aggressive behavior in birds reared in conventional systems can impact productivity due to territorial dominance on feed troughs, making it inaccessible for subordinate animals to access food without receiving aggressive pecks (Craig and Guhl, 1969). These animals may not receive the necessary nutrients for expected egg productivity and survival, and human monitoring seems complicated due to their eating frequency variability during the day. Additionally, the subtherapeutic use of antibiotics has been banned or discouraged regarding production systems (Mehdi et al., 2019). The primary concerns included transferring antibiotic gene expression and antibiotic resistance (Gadde et al., 2017; Medhi et al., 2018). Since antibiotic use is focused on disease prevention and promoting growth, there is an increased need to find alternative solutions for performance enhancers (Mookiah et al., 2013; Ferdous et al., 2019).

Prebiotic, probiotic, and synbiotic supplementation has become increasingly prevalent in experimental and agricultural settings in recent years. They are relatively low-impact supplements that would not harm the animals or strain the feasibility in economic expenses yet may provide health and productivity benefits. In recent years, different types of dietary supplementation have become more prominent in production settings. *Probiotics* are live (or, in some cases, heat-killed) microorganisms that support their host's intestinal health through homeostasis between beneficial and pathogenic bacteria (Pineiro and Stanton, 2007). The benefits of probiotic supplementation are well documented and include the prevention of pathogenic bacteria, improved digestibility, improved immunity, and positive behavioral changes (Desbonnet et al., 2010). Their mode of action varies to prevent pathogenic bacteria from proliferating and balancing the GI tract with more beneficial bacteria. Additionally, probiotics lower the intestinal tract's pH through their metabolites, competing for GI adhesion or nutrients (Mason et al., 2005) or stimulating the immune system (Ayed and Ghaoui, 2011; Pineiro and Stanton, 2007).

Tryptophan is an essential amino acid that is supplemented in the feed of animals and humans. It is a rate-limiting amino acid in protein synthesis, and it is involved in bacterial degradation (Höglund et al., 2019). This amino acid also synthesizes 5-HT, which is heavily involved in GI function (Cahir et al., 2007; Höglund et al., 2019). Serotonin is needed for normal GI functioning, such as mucosal secretion, peristaltic reflexes, and sensory responses to stimuli in the lumen. Therefore, Trp would also be essential to consider when supplementing with probiotics since it can serve as an enhancer of its beneficial effect through 5-HT availability. The gut microbiota impacts 5-HT expression through mediating Trp availability, and specific probiotics help in the expression of hormones, such as *Lactobacillus*.

Various studies with laying hens show how probiotic supplementation improves production parameters such as egg production, body weight, and overall nutrient absorption (Haddanin et al., 1996; Park et al., 2016). *Lactobacillus* strains are commonly used in poultry (Kalavathy et al., 2003; Lan et al., 2003; Lan et al., 2004; Sieo et al., 2005; Dec et al., 2014). A chickens' diet and its intestinal pH levels make it an ideal environment for the microorganism's survival (Lan et al., 2003; Ahmed et al., 2019). There is variability in the effects of probiotics on laying hens, but overall, it seems promising in feed conversion efficacy, body weight, and egg production (Lan et al., 2003; Lokapirnasari et al., 2019; Alaqui et al., 2020). Therefore, our study was conducted to understand the effect of Trp administration, with one of two microbiome altering supplements (probiotics or an antibiotic), on production performance and egg parameters of adult White Leghorn chickens.

Methods

All procedures involving live birds were approved by the University of Maryland Institutional Animal Care and Use Committee before initiating the experiment (#981251-1).

One hundred and forty-four female White Leghorn chickens (*Gallus gallus)* at 16 weeks of age were used for this study. Birds were marked with green or blue livestock markers and placed in wired layer cages hosting two birds per cage at the Animal facility building at the University of Maryland. The same protocol was applied to all birds for consistency in dietary and environmental background. A standard layer diet was administered until hens reached 90% lay (21 weeks of age). Food and water were given *ad libitum.* Thereafter, one of five treatment diets or control diets was assigned. Health checks, feeding, and egg collection were done daily.

Dietary treatments

Tryptophan was part of the basal diet at 0.16% (control). Additional Trp supplementation was added at 0.32% to a total dietary Trp of 0.48%. Microbiome altering treatments consisted of a probiotic mix (*L. acidophilus, B. bifidum, B. breve,* *B. infantis, B. longum, L. fermentum, L. casei, L. rhamnosus, L. plantarum*; 10^9 CFU/L or antibiotic administration (Erythromycin; 125 mg/L) both were added to the water (Table 1; 12 cages/treatment and 2 birds/cage). The cages were evenly assigned six treatments on a 2x3 factorial design and assigned to cage by randomized block design: 1) Control-control (control with no Trp supplementation; n=12); 2) Trp-antibiotic (Trp supplementation and antibiotic treatment); 3) Control-probiotic (no additional Trp with probiotic treatment); 4) Trp-control (Trp supplementation with no additional treatment); 5) Control-antibiotic (no additional Trp with antibiotic treatment); and 6) Trp-probiotic (Trp supplementation with probiotic treatment; n=12). Both probiotic mix and antibiotics were added to the water, which was changed daily. Birds were assigned to either of these five treatments and fed for 2 weeks.

Table 1. Dietary components of Trp, probiotic, or antibiotics treatments

Diet Treatment	**Trp supplement**	**Trp total**	**Probiotic supplement**
Control-control	0%	0.16%	No supplement
Control-antibiotic	0	0.16%	Antibiotic
Control-probiotic	0	0.16%	Probiotic mix
Trp-control	0.32%	0.48%	No supplement
Trp-antibiotic	0.32%	0.48%	Antibiotic
Trp-probiotic	0.32%	0.48%	Probiotic mix

Production performance and egg parameters

During treatment period, the body weight of the birds was recorded daily to obtain average BW. Additionally, egg weight and daily egg yield (total number of eggs produced daily x 100) were recorded. The average feed consumption was calculated by [(feed weight $_{initial}$ – feed weight $_{after\ 24hrs}$)- feed waste $_{tray}$]. Euthanasia and samples were taken after two weeks of treatment. After dietary treatment and behavioral recordings, birds were euthanized and eviscerated. Heart, liver, and fat pad weight was recorded (grams).

<u>Statistical analysis</u>

Analysis of the main effects and interaction of the tryptophan and probiotic treatments were done using the two-way ANOVA method. All data were tested for normality of distribution and variance. Log transformation was utilized when appropriate. Means of the data were analyzed by using a mixed model with SAS 9.4 software (SAS, Cary, NC) as a factorial arrangement of 2 x 3. Fixed effects in the model included main effects of tryptophan and probiotics and the interaction on body weight, heart weight, fat pad weight, FCR, egg weight, daily egg yield, and egg production. For ANOVA, P-values less than or equal to 0.05 were accepted as significantly different. The sample sizes for this production parameters section (n= 57) and egg parameters section (n=12), results shown in LS means + SEM, F-value, and p-value.

<u>Results</u>

Production performance

The effect of probiotic supplementation on body weight shows significant difference (Table 2). The control group had the highest body weight (1698.33 ± 39.7) compared to control-antibiotic treatment (1524.8 ± 43.5; $F_{5,\ 56} = 2.81$, $P = 0.03$). Heart weight in the Trp-antibiotic group was significantly higher (8.5 ± 0.4) compared to control-antibiotic birds (7.2 ± 0.5; $F_{1, 56}$ = 5.27, P = 0.03). The similar pattern was seen in fat pad weight where the Trp- antibiotic group was significantly higher (52.0 ± 6.4) than control-antibiotic (34.0 ± 6.4; $F_{1,56}$ = 4.99, P = 0.03). No significant changes were seen in liver weight ($F_{5,56}$ = 1.41, P =0.23) and FCR ($F_{5,30}$ = 0.84, P = 0.53).

Table 2. Overall means ± SEM for productivity after *Lactobacillus,* Erythromycin or control with or without additional Trp supplementation (grams).

Treatment	**Body weight +SEM**	**Heart weight+ SEM**	**Fat pad weight +SEM**	**Liver weight +SEM**	**FCR +SEM**
Control	1698.33 ± 39.7[a]	8.1 ± 0.4	58.8 ± 5.8	38.1 ± 1.7	1.9 ± 0.04
Control-antibiotic	1524.8 ± 43.5[b]	7.2 ± 0.5[b]	34.0 ± 6.4[b]	34.7 ± 1.8	1.9 ± 0.04
Control-probiotic	1587.38 ± 48.6	8.2 ± 0.5	52.2 ± 7.2	34.6 ± 2.0	1.8 ± 0.04
Trp-control	1538 ± 39.7	7.5 ± 0.4	48.6 ± 5.8	35.4 ± 1.7	1.9 ± 0.04
Trp-antibiotic	1653.2 ± 43.5	8.5 ± 0.4[a]	52.0 ± 6.4[a]	39.8 ± 1.8	1.9 ± 0.04
Trp-probiotic	1566.7 ± 43.5	7.6 ± 0.5	45.7 ± 6.4	34.9 ± 1.8	1.9 ± 0.04

Means within a column with different letters differ significantly (p <0.05)

Egg parameters

Results for overall egg production are shown (Table 3). There was no significant difference amongst treatments regarding egg weight ($F_{5,12}$ = 1.30, P = 0.33), egg production ($F_{5,12}$ = 0.49, P = 0.78), and daily egg ($F_{5,12}$ = 1.51, P = 0.26).

Table 3. Overall means ± SEM for egg parameters after either *Lactobacillus*, Erythromycin, or control with or without additional Trp supplementation.

Treatment	**Egg weight + SEM**	**Egg production + SEM**	**Daily egg yield +SEM**
Control	63.1 ± 1.2	98.1± 0.8	61.8 ± 1.2
Control-antibiotic	60.0 ± 1.2	98.6 ± 0.8	59.0 ± 1.2
Control-probiotic	63.1 ± 1.2	98.6 ± 0.8	62.1 ± 1.2
Trp-control	61.0 ± 1.2	98.6 ± 0.8	60.0 ± 1.2
Trp-antibiotic	60.6 ± 1.2	98.5 ± 0.8	59.7 ± 1.2
Trp-probiotic	60.6 ± 1.2	97.2 ± 0.8	59.9 ± 1.2

Means within a column with different letters differ significantly ($p < 0.05$)

Discussion

This study assessed the potential effects of Trp interactions with probiotics or antibiotics on production parameters in White Leghorn hens. Results show that treatment with antibiotics alone significantly decreased body weight. In contrast, when measuring fat pad weight and heart weight, supplementing antibiotic administration with an additional Trp increased weight on both organs. None of the dietary treatments caused a significant change in egg weight, egg production, and daily egg yield. These findings corroborated with a previous study where the probiotics supplemented did not impact production performance in poultry (Goodling, 1987; Huang et al., 2004; Kurtoglu et al., 2004; Panda et al., 2008).

Results show a significant effect on body weight, antibiotic administered group weighed less than controls. Nonetheless, probiotic treatment with different strains appears to cause marginal differences or none (Goodling et al., 1987; Kurtoglu et al., 2004; Panda et al., 2008). Further studies should be done to recognize if the changes, or lack of, in body gain are associated with any influence in the animal's carcass yield. This observation is significant when applying results to the industry, as the focus on body gain should be on areas that are commonly processed for meat and not reallocated weight across unwanted parts of the body.

There were no significant differences in the egg parameters measured in this study: egg weight, egg production, and daily egg yield. Previous studies show how probiotic supplementation contributes to improved egg production (Abdulrahim et al., 1996; Panda et al., 2008; Mikulski et al., 2012) and egg quality (Ribeiro et al., 2014). However, in a study with a similar Lactobacillus probiotic by Goodling et al. (1987), no effect took place on egg production and egg weight in the laying hens. Similarly, egg weight, feed efficiency, and bodyweight layers were unaffected by commercial probiotic supplementation in a study by Kurtoglu et al. (2004). Data from several studies with laying hens and different strains of probiotics show no significant differences in egg production with treatments (Miles et al., 1981; Mahdavi et al., 2005; Ramasamy et al., 2009). Variance in the effect of probiotics is attributed to differences in strains, effectiveness, and interactions with the individual's set of diets and environment (Nahashon et al., 1994). More importantly, the probiotic must be viable to carry out their beneficial effects (Nahashon et al., 1994). Often, at the time of supplementation, many microorganisms in a probiotic mixture have lost most of their beneficial qualities.

Commercial poultry diets vary between farms and whether the animal is used for meat or eggs. However, all diets contain limiting amino acids to suffice dietary demands (Fouad et al., 2016; Jiang et al., 2019). Data shows that birds should be fed a diet containing at least 0.18% Trp (Cardoso et al., 2014). This amount corresponds to having the necessary amount of Trp to express its capacities of lipid metabolism and another nutrient breakdown (Rogers and Pesti, 1992). Our study focused on additional Trp than the minimum requirements, and changes were not as defined as its deficiency. It is essential to mention that Trp is involved in many processes, and its bioavailability depends on whether it is metabolizing for melatonin, ghrelin, 5-HT, kynurenic acid, or quinolinic acid (Le Floc'h et al., 2011). Additionally, other gastrointestinal processes come into play during Trp utilization.

` Many of the common probiotics used, especially in poultry, are microbial strains commonly found in the animal's gut microbiome. The chicken's gut microbiome is involved in nutrient breakdown, growth and development, immunity, and the hosts' wellbeing (Shakouri et al., 2009). Dietary composition plays a crucial role in the success of these processes, as these microorganisms need it to produce the necessary metabolites. Therefore, dietary changes could affect the integrity and composition of this organ. Previously, we mentioned the influence of the gut microbiome in hormone expression and monoamine production, such as 5-HT, through the utilization of the amino acid Trp. Lactobacillus, Enterococcus, and Bifidobacterium are some of the bacteria used for commercial probiotic use (Khattak and Helmbrecht, 2019). There are different proportions of beneficial and pathogenic bacteria on an individual level. However, often, since chickens tend to stay in one same environment, with similar conspecifics through the rest of their life, this microbiome composition can be similar. This quality could be beneficial when trying to manipulate the gut microbiome composition. Further studies should focus on doing a gut microbiome assessment of the chickens after probiotic supplementation to understand the impact of probiotics on the gut microbiome's components.

Since various conditions are responsible for the success of the probiotics, when supplemented for the effect of nutrient digestibility, it may not always give the expected results. In finishing pigs, Bacillus supplementation did not improve nutrient digestibility (Chen et al., 2006). Although our study did not test nutrient digestibility, this digestive capacity correlates with the animal's ability to use the nutrients for overall weight and productivity. Additionally, different from probiotics but potentially beneficial, antibiotic use has improved the microbial community of the gut microflora through short-chain fatty acid (SCFA) production and its anti-inflammatory effect (Choi et al., 2018). Our results demonstrated an effect of probiotic supplementation in higher liver weight and heart weight, potentially pointing towards that SCFA's production capacity.

The chickens used in our study had reached sexual maturity at the start of the treatments, so manipulating the integrity of the gut microflora proved more challenging. The reason for this is that the gut's microflora is established early in life, and manipulations to it have a more impactful effect during this early time in life (Yeoman et al., 2012). Supplementing a Lactobacillus fermentation product to laying pullets at different concentrations had no significant effect on production parameters like body weight, egg production, or feed efficiency (Cerniglia et al., 1983). Similar results were seen in Goodling et al. (1987) with laying pullets. To further explore bird age's potential effect on probiotic efficacy, Nahashon et al. (1996) supplemented a mixture of Lactobacillus and molasses to pullets from the growing phase to the laying phase. This supplementation improved feed consumption and BW in the growing phase, and in the laying phase, it also improved egg size and calcium retention. Nonetheless, this egg quality may or may not be beneficial in a production setting as it depends on the final product and the industry in question.

This study suggests that Trp, probiotics, and antibiotics interact with the gut microbiome, impacting weight measures in laying hens. Neither Trp nor probiotics had a negative effect on production. Further research is needed to optimize supplementation for production and animal welfare. Bodyweight, liver weight, and heart weight were affected by the interactions between treatments and the gut microbiome, showing the impact of supplementation in production parameters.

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Chapter 4: A discussion of the effects of Trp administration, its interactions with probiotics or antibiotics on laying hen's behavior, physiology, and overall well-being

Promoting positive animal welfare is crucial in animal husbandry. However, implementing practices that foster positive welfare can be more complex in different production settings, as it depends on the animal in question and the degree of concern given to its rearing method (Fraser, 2014). In poultry husbandry, especially egg production, there is consumer concern about overcrowding in housing conditions, the lack of environmental enrichment, and as a result, the expression of maladaptive behaviors. Additionally, productivity can be impaired if severe or aggressive pecking leads to increased energy needs, resource guarding or high levels of cannibalism (Broom, 2000). Our study assessed the behavioral and production effects of dietary treatments, specifically, if additional Trp could promote positive behaviors and if probiotic supplementation facilitated this expression. Regarding physiology, Trp and probiotic interactions could improve production parameters.

The results of our study showed that Trp administration with probiotics did alter behavioral displays. Birds sat less while stood and ate with more frequency. Probiotic supplementation impacted other comforts and social behaviors such as preening and gentle feather pecking (GFP), where preening was reduced, and GFP increased. Although both behaviors require manipulations of the feathers, animals engaged in social grooming for the most part. Overall, good welfare in a dynamic production system balances positive and negative behavior since the animals are exposed and reacting to a changing environment.

Probiotic supplementation can impact regions of the brain involved in emotional processing (Tillish et al., 2013; Bagga et al., 2018). The treatments in our study impacted behaviors in layers, where probiotics and Trp interactions increased sitting, walking, and eating behavior. Additionally, probiotic supplementation alone also impacted other behaviors like standing, preening, and gentle feather pecking. Further studies are needed to understand the mechanisms of interaction between the Trp and microbiome altering treatments and the potential role of the serotonergic system. It is essential to better understand the biochemical pathway of 5-HT in the gut microbiome of birds through Trp and probiotic supplementation.

Dietary supplementation is not a new practice in chicken production; research about its impacts has increased in recent years (Khan and Naz, 2013; Untoo et al., 2018; Hernandez et al., 2019; Ramlucken et al., 2020). The field of commercial poultry is undergoing a process of innovation, with increased attention to animal welfare. Its goal is to slowly transform rearing procedures for the benefit of the producer, the consumer, upholding demands and without negatively affecting the animal (Khan and Naz, 2013). Probiotics can alter the microbiota composition and integrity. So, probiotics have been stated as a possible supplementation to reduce gut microbiota problems, resulting in better expression of behavior-related neurotransmitters. Although it is not simple to set forth all the changes that prove advantageous for production animals, the addition of these beneficial bacteria to their diet could help ameliorate negative behaviors.

Although previous research shows how manipulating the neurotransmitter 5-HT's expression through Trp supplementations affects behavior, other circumstances can prevent the onset of behavioral expression (Dawkins, 1990). Some reasons could be the animal's interactions with other conspecifics, the rearing environment (lack of environmental enrichments), human disturbances at the time of feeding, or internal motivation to express such behaviors. Additionally, Trp metabolism and probiotic absorption hold a crucial role in this expression, directly and indirectly. Trp metabolism is associated with bacterial degradation, which competes with other existing microorganisms, including probiotic supplementation (Liu et al., 2015).

Serotonin production comes from the chemical breakdown of anaerobic fermentative bacteria (Fukumoto et al., 2003). Through the release of SCFA, they induce the production of 5-HT through EC cells. Specifically, Lactobacillus aid in Trp mechanisms (Villarubias, 2015). These findings illustrate some of the many reasons for integrating probiotic supplementation into poultry production systems. The successful implementation of probiotics depends on the strain and interaction with the hosts, dose and frequency of supplementation, the host's age, stress level, and individual genes (Bomba et al., 2002; Chen et al., 2006). Moreover, the gastrointestinal microflora of chickens is not fully understood (Yeoman et al., 2012). Every chicken possesses a unique intestinal set of microbial organisms that form that community, even when these birds are fed the same diet, have the same age, and are raised under the same conditions (Shakouri et al., 2009).

Prebiotics and probiotics supplementation improved egg quality in laying hens (Yousefi and Karkoodi, 2007). Our results showed no differences in egg parameters, but some patterns of interest were traced regarding weight differences in the birds. Bodyweight was reduced in groups supplemented with antibiotics alone; liver and heart weight were higher in birds supplemented with antibiotics and additional Trp supplementation than control and control-antibiotic groups. These results are not unique to our study as data from several studies with laying hens, and different strains of probiotics show no significant differences in egg production with treatments (Miles et al., 1981; Mahdavi et al., 2005; Ramasamy et al., 2009). Commercial poultry diets vary between farms and whether the animal is used for meat or other by-products, but they all contain limiting amino acids to suffice dietary demands (Fouad et al., 2016; Jiang et al., 2019).

Our research sought to determine if findings of studies performed on humans and other mammals can also be applied to birds, given that these are also omnivores. It is important to note that the digestive system of birds is different, and consequently, they can yield different results. Nonetheless, animal well-being should remain the priority as it unequivocally affects all other areas of animal production. To conclude, a research setting is very different from a production setting. It has a more controlled environment, more immediate goals, and a set of parameters established. These differences hinder the potential applications of results in the field. Ideally, further studies should be conducted in settings more similar to a typical production facility, which would allow for a more accurate assessment of the influencing parameters in the development and physiology of laying hens. Our studies reveal that Trp, probiotic, and antibiotic treatments interact to cause changes in behaviors and production.

Appendix I – Behavioral Testing Ethogram

Table A1. Ethogram used for home cage focal behavioral testing.

Behavior	Description
Stand	Upright position with both feet touching the ground
Sit	Body is touching the ground with bent legs and eyes opened
Walk	Taking more than one step forward
Eat	Head in feeder and while manipulating or ingesting feed
Drink	Beak in water from water source
Preen	Self-manipulation of feathers with beak
Peck cage	Pecks directed to the walls of the cage
Wing flapping	extension of wings followed by flapping movement
Gentle feather pecking	Pecking at conspecifics' feathers in a social or investigatory manner

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