# The bacteria-gut-brain axis: Gut bacteria as a key regulator of social stress and stress-related injurious behaviors in chickens

Sha Jiang[1], Jiaying Hu[2], and Heng-wei Cheng[3*]

*Correspondence should be addressed to: heng-wei.cheng@ars.usda.gov (H-W. C.)

[1]College of Animal Science and Technology, Southwest University, Chongqing, P. R. China, 400715

[2]Purdue University, West Lafayette, IN, USA, 47907

[3]USDA-Agricultural Research Service, Livestock Behavior Research Unit, West Lafayette, IN, USA, 47907.

**Simple Summary**: Exhibition of injurious behaviors, such as injurious pecking and cannibalism, are a serious welfare issue with devastating economic consequences to the global poultry industry. These harmful behaviors occur in all current housing systems, leading to suffering and death of laying hens without beak trimming. Beak trimming, both hot blade and infrared, has been used as a common practice for preventing or reducing injurious behaviors in laying hens. However, there is a considerable body of morphological, neurophysiological, and behavioral evidence demonstrating the emergence of markers of acute, chronic pain or both as a result of trimming. Our investigation outcomes indicate that probiotic supplementation can be used as an alternative to beak trimming to reduce injurious behaviors in hens via regulating the bacteria-gut-brain axis. As a result, it will have a high impact factor for poultry production practices. The novel approach could be transferred directly or indirectly to other species of farm animals that are subjected to painful husbandry procedures, such as dehorning of calves and teeth-clipping of piglets for preventing body injuries.

**Abstract:** Some management practices, such as maintaining birds under high group density, used in the poultry industry may cause birds stress, leading to injurious behaviors, such as injurious pecking, aggression, and cannibalism. In addition, some management practices used to prevent severe injuries in birds may cause pain. Beak trimming (BT), removal of 1/3 to 1/2 of a beak, is a routine husbandry procedure practiced in laying hens to prevent or reduce injurious behaviors. However, BT causes tissue damage, which may increase somatosensory sensitization of the damaged nerve tissues, resulting in pain (acute, chronic or both) in the treated birds because the beak is a complex, functional organ with an extensive nerve supply. BT has already been heavily regulated or prohibited in several European countries and, in time, this trend will impact the practice used in the United States poultry industry. With the growing public concern for poultry welfare there is a pressing need to identify and develop alternatives to BT. Probiotics defined "as a source of live (viable) naturally occurring microorganisms (direct-fed microbials)" have been used as dietary supplements or functional foods to target gut microbiota (microbiome) for prevention or therapeutic treatment of mental diseases including social stress-induced psychiatric disorders in humans and various experimental animals. In our studies, chickens were used as an animal model to assess if dietary supplementation of probiotic, *Bacillus subtilis*, reduces injurious behaviors following social challenge. Hens of Dekalb XL strain, an aggressive line, were used in the studies. Our results indicate that dietary supplementation of the *Bacillus subtilis* based probiotic reduces aggressive behaviors in chickens. These results suggest dietary probiotics could be a suitable strategy for increasing hosts' health status and welfare conditions.

**Keywords**: laying hen, social stress, injurious behavior, microbiota, probiotic, bacillus subtilis

## 1. Introduction

Commercial chickens have been selected for production (laying hens for eggs and broilers for meat) to meet the increasing demand for poultry products. The breeding programs, however, subject chickens to physiological dysfunction and immunosuppression by overloaded on reproduction or growth, subsequently increasing susceptibility to metabolic disorders and management-associated stressors [1, 2]. For example, a hen of egg-laying breeds produces approximately 310 eggs annually with a low feed consumption of just 110 grams per day. The extreme selection for one trait (production) could affect other biological traits, causing negative impacts on animal health and welfare, such as aggression [3]. Selection for production may increase aggression as that from an evolutionary point of view, aggression in animals is a natural behavior associated with competition to deal with life-threating situations affecting an individual's survival, growth, and reproductive success within a group [4, 5].

Over the decades, the managements and production environments for chickens have undergone dramatic changes. As production grows to large-scale operations to increase profiles, the housing systems and management practices in the modern poultry industries have often been designed based on economic and ergonomic aspects (cost, space, and workload); and chickens are often reared in a large group at a high density. However, there is a trade-off between profiles and animal health. Based on the "frustration-aggression hypothesis" [6, 7], aggression is a predicable reaction to external stimulations. Chickens reared in a large group may have a great risk of instability of social hierarchy (social dominance order), resulting in social stress and stress-related injurious behaviors [8].

Additionally, some management practices used in the poultry industry may cause chickens stress. In routine practices, chickens are repeatedly transported to new environments and mixed with unfamiliar ones (social disruption) during their lifetime, such as transferring laying hens from grower to layer facilities [9, 10]. Furthermore, the conventional (battery) cage system is the most common housing facility for laying hens in the United Sates (U.S.): an estimated 85% of the commecial eggs are derived from the caged hens [11]. The high stocking density of hens and limited space for hens to display their "natural" behaviors (such as foraging, exploration, perching, and nesting) negatively impact their welfare status, resulting in a chronic state of stress [12]. Chickens enter a 'pre-pathological state', showing physiological and metabolic disturbances, exhibiting abnormal behaviors (feather pecking, aggression, and cannibalism) when they are unable to cope with these management practices [13, 14]. Social stress is a major concern in all current housing environments including cage and cage-free systems [15]. Especially, stress-induced disruption of intestinal bacterial diversity causes inflammation and or infection locally and systemically [16, 17], by which it further affects hosts' mental state and behaviors via the bacteria-gut-brain axis [18, 19].

Intestinal bacteria, function as a virtual endocrine organ across species, release various bioactive compounds playing an important role in brain function in regulating stress response and related behavioral health [20, 21]. Numerous studies have evidenced that there is an intimate link between the gut bacteria and animal social behaviors, that is the gut bacteria influencing hosts' social behaviors, while the inter-group social structure shaping the composition of the gut microbiota [19]. Chronic social stress disrupts gut homeostasis, increasing intestinal permeability (leaky gut), and breaks the linkage, causing neuroinflammation, consequently leading to mental disorders and related abnormal behaviors [22-24] such as aggression [25, 26]. Recent studies have reported that dietary supplementation, such as probiotics, is a useful method to suppress or prevent mental disorders via regulation of the microbiota-gut-brain axis in humans [27, 28] and rodents [29, 30] (Figure 1).

Probiotics (also called psychobiotics or bio-friendly agents) define "as a source of live (viable) naturally occurring microorganisms (direct-fed microbials, DFMs)" with their functions in immunomodulation [31], metabolic homeostasis [32, 33], and neuroendocrine regulation [21, 34]. Probiotics, such as *Bifidobacterium* and *Lactobacillus* species, have been used as dietary supplements to target gut microbiota (microbiome) for a novel promising therapeutic approach of various diseases, including social stress-induced mental disorders, via regulation of the microbiota-gut-brain axis in humans and various experimental animals [27-29, 35]. The investigation outcomes indicate that the effects of probiotics on physiological homeostasis, immunity, stress resistance and related health status are affected by multiple factors, including the probiotic species, its concentration and duration as well as the host's age and health status [36-39].

*Bacillus subtilis* is one of the three most common species of the commercial probiotic products (the other two: *Lactobacillus acidophilus* and *Enterococcus Faecium*) currently used in humans [40-42] and various animals [43-45] including chickens [46, 47]. In chickens, similar to it in other species, *bacillus subtilis* has been used as a growth promoter and or an dietary antimicrobial alternative for increasing production under both normal and stressed rearing conditions via regulation of intestinal biological and morphological functions to inhibit inflammation and infection [42, 48-51]. In our studies, we also reported that *Bacillus subtills* improves performance [52], meat quality [53-55], immunity [52], and skeletal health [56] in broiler chickens under chronic heat stress. The objective of this review was to focus on the potential use of *Bacillus subtilis*-based probiotics to prevent or reduce injurious behaviors, i.e., feather pecking and aggression, in laying hens.

## 2. *Production Environments of Laying Hens*

Chickens as well as other farm animals are constantly selected by both nature (natural selection) and humans (artificial selection). During the selections, chickens' biological and behavioral characteristics are constantly changing sensibly and/or insensibly. The process is affected by multiple factors, including their surrounding environments; by which the animals are selected for increasing their fitness (that is survival and reproductive success) over generations.

In the wild animal kingdom, chickens often live in small groups, consisting of a single rooster and several hens with chicks. Chickens live for their own needs, but battle with multiple dangerous issues, such as natural predators, disease (parasites, inflammation, and infection), inclement weather changes (cold and hot), and unpredictable food and water supply. Although chickens can perform all their inherited (species-specialized) and learned (individual-specialized) behaviors in order to meet their physical, physiological, and behavioral needs, a natural environment may not be the best choice of animals. Over the past several decades with scientific discoveries and technological advances, farming practices were shipped from the backyard farms to the intensified and specialized industries to increase economic profits and to meet the increasing demand for food of the expanding size of the human population. In the artificial farming, chickens live for humans' needs including food (eggs and meat). Compared to their native partners, industrialized farming provides for the chickens' needs, such as food, water, shelter, and protection from weather extremes and predators via modifying the birds' living environments, with a goal of best accommodating both human and animal needs. However, the housing conditions and related management practices may influence chickens' physiological and behavioral health and welfare if the animals cannot adapt to the environments [57].

Currently, the conventional (battery) cage system is the most common housing facility for laying hens in the U.S. and most non-EU countries, estimated 85% of the commercial egg production in the U.S. and 90% of the world's egg production [11]. Typically, laying hens are housed in groups ranging from five to nine birds per cage or greater at a density of 67 – 86 $in^2$/hen, starting at about 18 weeks of age. The main disadvantages of the conventional cage system are 1) discomfort, resulting from limited space for hens to move (walking and running) and rest (standing, sleeping) and without resources for hen to perform roosting, dustbathing, nesting, and foraging; and 2) increased body injury, resulting from feather pecking, aggression, and cannibalism [58, 59].

One of the possible ways to improve hen health and welfare is to modify their rearing environments, and several alternatives to the conventional cage system have been developed, such as enriched cage (consisting of a nest, letter bath or scratch area, perches, and abrasive strip) and cage-free systems with or without outdoor access such as aviaries (single- and multiple-tiered). Although hens housed in the enriched cage system and non-cage systems seem to be a possible ways to improve their welfare by displaying some degree of natural behaviors, such as nesting, roosting, and scratching [60, 61], there is a high risk of increase in frequency of exhibiting of injurious behaviors, resulting from large group sizes and social instability [62-64]. Currently, approximately 230 corporate customers have pledged to buy eggs only from cage-free hens [65], which may further impact bird health and welfare based on the acute welfare status of laying hens in the alternative systems. Injurious behaviors become even more serious with some countries of the E.U. to ban on the using of conventional cages and BT [66, 67]. Cronin et al. [10] reported that once initiated, severe feather pecking spread via social learning in hens reared in free-range egg production, and by 40 weeks of age, approximately 98% of hens had plumage damage.

## 3. Injurious Behaviors in Laying Hens

Chickens as well as other farm animals were domesticated from wild animals several thousand years ago. During domestication and subsequent artificial selection (breeding), the selected animals continuously change their biophysiological stress response (adaptive capability, i.e., effect of reactive ability on growth, metabolism, and reproduction) and biobehavioral profile (social and emotional exhibitions as well as cognitive ability) to 'fit' the given environments and related management practices. Alternatively, not all individuals and species (genotype and/or phenotype) of animals have equal capability to adapt to their environments or modify their physiological and behavioral characteristics (capability of biological plasticity) in response to environmental challenges. Consequently, the animals exhibited less adaptation may carry a risk of poor health by reducing fitness, resulting in distress.

### *3.1. Feather Pecking*

Feather pecking (FP) in laying hens is a behavior performed by birds pecking repetitively to conspecifics. It includes two categories: gentle and severe FP [68], driven by different motivational systems[69]. Gentle FP, repeated pecking at the tips and edges of feather without removal of the feather from the receivers, has been considered as a common behavior related to social discrimination and exploration [69, 70] without association to severe FP [71]. 0thers suggest that gentle FP acts as a precursor of severe FP [68] and associated with plumage damage [68]. Severe FP, forcefully pecking at and pulling of feather, is redirected foraging behavior [68, 72], which is significantly increased if hens' foraging motivation cannot be fulfilled [73], such that suitable substrate is no provided [74] or removed [75]. FP, especially severe FP, is a serious health and welfare issue in the poultry egg industry, which affects up to 80% of birds in all current housing systems [76]. FP in extreme cases leads to cannibalistic pecking (cannibalism), removing and eating flesh from the victims, which can be further enforced via the gut-brain reward system (the central serotonergic and dopaminergic systems) and spread among the conspecifics (as a socially transmitted learning behavior), leading to death [77]. Cannibalistic pecking can be also induced by aggressive pecking following skin damage.

### *3.2. Aggression and Cannibalism.*

Aggression within a group is to establish a dominance hierarchy when the animals are first brought together in a common environment [5]. In chickens, aggressive pecking, directly to the head and comb area, occurs to establish hierarchy within a group or in response to stress and related social instable during certainly rearing practice, such as transferred birds from grower to layer facilities. In an evolutionary view, aggression (also called combativeness) in animals, as a part of natural behaviors, is related to survival, growth and reproduction [4, 78, 79]. Based on the natural selection theory, an animal's productive success is correlated with its competitive ability. Traditional techniques used for selection of animals for breeding are based primarily on individuals with high productivity, resulting in a high risk of impaired ability to copy with their environments and related biological problems, increasing competition and aggression [80]. In addition, based on the "Frustration-aggression hypothesis", aggression is a predicable reaction to external stimuli [81, 82]. When restrictive environments, such as conventional cages, do not allow chickens to perform their natural behaviors, they enter a state of frustration with stress reactions and increased aggression [83]. In poultry, for example, egg production has been increased through selection while potentially injurious feather pecking, and cannibalism have concurrently increased. For instance, our previous studies have revealed that through more than 20 years of selection, egg production has been increased significantly in a former commercial Dekalb XL strain reared in conventional cages, whereas mortality due to aggression and cannibalism in non-beak trimmed hens has also increased about 10-fold [80, 84, 85] (Figure 2).

## 4. Beak Trimming in Laying Hens

In response to growing pressures, management practices of laying hens have been modified and various dietary supplementations have been provided in attempts to prevent social stress and stress-associated injurious behaviors. For instance, the modifications include reducing light intensity, changing the nutritive value or taste of diets [86], providing straw or grain [87] or pelleted diets [88], housing hens in floor-pens [89], and developed enriched cages [90, 91]. However, these methods have had limited success and provided no guarantee of preventing these injurious behaviors.

Beak trimming (BT. also termed debeaking, beak tipping, beak mutilation, and partial beak amputation) is a routine procedure practiced in the U.S. egg industry and in the most non-EU countries for preventing or reducing social stress and stress-related injurious behaviors, i.e., feather pecking, aggression, and cannibalism. The process involves use of either infrared (at the hatchery) or hot-blade (prior to 10 days of age) treatment to removal of a portion of the upper, or upper and lower, mandibles (i.e., 2-3 mm the remained length of the upper beak distal from the nostrils, or up to one third of either mandible)[11]. A beak is a complex, functional, and highly nerve fibers innervated organ that contains great amount of various sensory receptors including mechanoreceptors, thermoreceptors, and nociceptors [92]. Worldwide, BT continues to solicit a great deal of debate pertaining to the relative impact of BT on bird welfare. While the bestowed benefits of lowered aggression, feather pecking, and cannibalism may indeed favor improved welfare during the laying cycle [91], there are considerable anatomical, physiological, and biochemical changes occur in cut peripheral nerves and damaged tissues[92], criticizing it causes pain (acute, chronic or both) with negatively impact on the welfare of billions of chickens annually. There is growing pressure from animal welfare and consumer groups advocating the banning of this practice and to develop alternatives. Although injurious behaviors can be improved through genetic selection such as group selection in which social interaction is included [93, 94]. However, there is "*no sign that breeders will be able to guarantee the 'non-peck' layers in time*" [95] in the near future. Therefore, an obvious solution is to develop a welfare-friendly alternative to BT that minimizes social stress, thereby preventing FP and cannibalism.

## 5. Stress and its Regulation Systems in Laying Hens

### *5.1. Stress.*

Stress has been simplified as a body's reaction (physiological and psychological) to real or perceived harmful or danger situations. Naturally, stress is a part of an animal's daily life, which, at certain levels, will enhance its adaptability (general adaptation) and survivability (health adjustment). However, an excessive reaction could cause an animal's physiological and or psychological damage (stress syndrome), such as exposed to uncontrollable, unpredictable (lack of coping availability), and or prolonged stress (chronic stress). In humans, emotional susceptibility in an inter-group is associated with individual differences in the functions of the hormonal and neurochemical systems in response to internal and external stimulations [96, 97]. Although there are dissimilarities between humans and chickens, as indicated, the neural circuitries for stress reaction appear to be evolutionarily conserved across the vertebrates [98]. Previous studies have reported that birds' brain possess a core "social behavioral network" which is humongous to the social behavioral network of mammals [99]. It has also evidenced that the central nuclei involved in moodiness in avian, at least in part, are morph functional homologous to the mammalian nuclei [100], such as the hypothalamus [101], nucleus taeniae (homologue to the amygdala of mammals) [102, 103], and raphe nucleus [104]. These nuclei exert a similar cognitive abilities and consciousness [105] with capability of plasticity in response to environmental stimulations [106]. In addition, there are similar distributions of neurotransmitter receptors, including serotonergic receptors, between birds and mammals [107, 108]. Similarly, stress in chickens acts the two main stress response systems, the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-medullary-adrenal (SMA) axis. The function of the HPA axis, as a neuroendocrine system, mediates a longer duration accumulated stress response, while the activity of the SMA axis drives a short-term "fight or flight' reaction. Alterations of neuroendocrine homeostasis, i.e., corticosterone (CORT, regulated by the HPA axis) and catecholamine (dopamine, DA; epinephrine, EN; and norepinephrine, NE, released from the SMA), have been identified as the final common pathways in controlling animal pathophysiological status and behaviors [109].

*5.2. Corticosterone*.

CORT is produced by the adrenal glands in response to stimulations, and plasma CORT concentration has been used as a peripheral marker of physiological coping ability. For example, territorial aggression in birds is correlated with the condemnations of CORT [110]. Through binding to their brain receptors [111], elevated CORT affects neuronal biological functions, including neurotransmitter systems, such as serotonin (5-HT) biosynthesis, metabolism, and receptors in both the raphe nucleus and the hypothalamus [112, 113]. Both raphe and hypothalamic circuits control aggression in humans and rodents [114-116]. In rodents, for example, elevated activity of the HPA axis, exhibited as increased corticotrophin releasing hormone (CRH) expression and circulating CORT levels, are correlated with anxiety-like behaviors [117, 118] and cognitive dysfunction-associated with neuropsychiatric disorders [119]. Similarly, exposure to excess glucocorticoids during chicken embryonic development (E11) increases the frequency of aggression at postnatal week 3 through alterations of the functions of the HPA axis and the serotonergic system [120].

*5.3. Dopamine***.**

Numerous studies have evidenced that DA involves in regulation of aggressive and defensive behaviors, and DA concentration has been used as an indicator of stress response [121, 122]. The midbrain/striatum DA storage capacity is negatively correlated with aggression in humans [123] . In addition, changes in DA concentrations in selected brain regions, such as the hypothalamus, have been found during and following aggressive and defensive behaviors in both humans and rodents [124, 125]. Mice that exhibit isolation-induced aggression have higher levels of DA in the nucleus accumbent [126], and aggressiveness of mice can be decreased or prevented by DA blockers [127] . Great concentrations of DA are also found in the brain of Japanese quails with aggressive behavior [128]. The D2 receptor gene represents one of the few single-site loci associated with abnormal behaviors in humans, and thus it may also be linked to abnormal behaviors in hens, such as stereotyped cage pecking and/or feather pecking [129]. In addition, aggressiveness can be increased by pharmacologically activity the D2 and D1 receptors with agonists such as apomorphine and dihydrexidine, respectively [130].

*5.4. Epinephrine and Norepinephrine*

Both EN and NE, as CORT, are known as “stress hormones”. Previous studies have shown that EN and NE, as indicators of well-being, are involved in controlling behavioral expression and linked to mood control (fight or flight)[131] and stress adaptation, such as post-traumatic stress disorder [132] and stress-induced neuroinflammation in major depressive disorder [54]. In experimental animals, plasma NE can be elevated by acute stress, and the degree of increase reflects the intensity of the stressful stimuli. Clinical and experimental studies indicate that increased turnover of NE in the hypothalamus is correlated with increased serum levels of CORT [133, 134] and anxiety-like behaviors [135]. Dysfunction in NE system as well as 5-HT system has been found in psychiatric disorder [136] and the serotonin-norepinephrine reuptake pathway has become a target for psychotherapy of posttraumatic stress disorder [137].

*5.5. Serotonin.*

The brain serotonergic system plays a critical role in regulating behaviors, especially aggression [138-140], which is crossed between vertebrates and invertebrates [141]. Tryptophan hydroxylase 2 (TPH2), rate-limiting enzyme of 5-HT synthesis in the brain, has become a therapeutic target for psychiatric disorders [142]. In addition, concentrations of 5-HT and its metabolites as well as the density of 5-HT receptors have been used as major indicators of displaying aggressive behaviors in experimental animals [143-145]. Aggressive animals have lower levels of 5-HT in the brain, including the hypothalamus [146], while experimental increase of 5-HT and/or 5-HIAA in the brain (the lateral hypothalamus and amygdala) blocks or retracts killing behavior in rodents [147]. In human studies, administrations of compounds that specifically elevate 5-HT levels in the brain (e.g., Prozac) have been used effectively to treat depression and associated behaviors. Similarly, supplemental tryptophan decreases social-mixing stress and stress-related aggression in pigs [148], feed-restricted male chickens [149], and injurious pecking in laying hens [150, 151]. In addition, previous studies have shown that the functions of 5-HT1 and 5-HT2 receptors are linked to aggression [152, 153]. Aggressiveness has been demonstrated to increase with impaired receptor functions [152, 154] or by knockout of 5-HT1 receptor genes [152]. S-HT has been used as a biomarker for selecting kind and gentle laying hens, with an aim to eliminate BT [143, 155-157].

Previous studies conducted in our laboratory have reported chicken strain differences in social reactions through genetic selection programs. The DXL hens have distinct stress levels in attempting to adapt to their social environments [158, 159] and exhibit different levels of aggressiveness [3]. In addition, the neurotransmitters, 5-HT and DA, and the function of the HPA axis are regulated differently between selected chicken strains, mean bad birds (MBB) and kind gentle birds (KGB) [3, 159]. MBB is a high aggressive strain selected for both low productivity and low longevity, resulting from injurious pecking and cannibalism; while KGB is a low aggressive strain selected for both high productivity and high longevity. There results suggest that injurious behaviors and related social sensitivity of chickens, like that in mammals, are regulated via the serotonergic system and the HPA axis [120, 160, 161]. The mechanisms underlying aggression in laying hens may be analogous to those described in humans and rodents [145]. Stress-caused destruction of intestinal bacteria disturbs the bilateral connection of the microbiota-gut-brain axis in chickens, affecting physiological homeostasis and behavioral exhibition [24, 162, 163].

## 6. Gut Bacteria and the Microbiota-Gut-Brain Axis

*Gut microbiota and the gut-brain axis*.

Gut microbiota resembles an endocrine organ and responds to various internal and external stimuli to regulate hosts' health status through integrating the signals received from the metabolic, immune, endocrine, and neural pathways via bidirectional communication of the gut-brain axis [20, 21, 164, 165]. Various psychological (mental status) and/or physical (environmental conditions) stressors alter gut microbial composition; and the inability to maintain a healthy gut microbial profile is associated with alterations of the host's behaviors and health status [25, 35, 166]. In humans, change of gut microbiome is associated with depression and neuropsychiatric disorders caused by neurodegeneration or neuroinflammation [167-170]. Similarly, gut microbiota (microbiome) in chickens affects birds' health [171-173]; and the gut microbiota diversity is different in layer strains selected for high and low feather-pecking [151].

The mechanisms of gut microbiota effects on hosts' stress response are not fully understood, but could be related to stress-caused anatomical and functional disorders of the gastrointestinal tract (GIT) by: 1) disrupting the commensal bacterial populations and colonization (the stability of the gut microbiota), thus reducing beneficial bacteria and increasing pathogens (low-grade inflammation); 2) increasing pathogens survival and innovation capability (bacterial translocation to increase neuroinflammation); 3) disrupting absorption of nutrients and minerals, resulting in metabolic disorders; 4) disrupting microbial neuroendocrine functions (producing several signaling molecules and neurochemicals including 5-HT in the GIT; 5) disrupting the gut epithelial barrier, thereby increasing intestinal permeability causing the gut to leak certain bacteria (leaky gut), resulting in systemic inflammation and or infection; 6) damaging epithelial cells, thus producing free radicals and reducing antioxidant efficacy (oxidative stress); and 7) interrupting intestinal integrity, thereby leading to intestinal inflammation [174-178]. These changes in gut microbiota influence hosts' behaviors and health status, via the peripheral nerve systems (the vagus nerve, enteric nerve, and autonomic nervous system), hormone signaling, the immune system, and microbial metabolites (short chain fatty acids) [34, 179] to regulate pathophysiological functions of the microbiota-gut-brain axis. For example, intestinal bacteria are involved in tryptophan metabolism [180-182]. Tryptophan, a precursor of 5-HT, directly affects brain 5-HT synthesis as tryptophan can pass the brain-blood-barrier [183-185]. Tryptophan has long been used to attenuate aggressive behavior, control stress, and modulate immune function in humans as well as several species of farm animals including chickens. Animals (such as pig, rat, and chicken) fed tryptophan-enriched diets (neurodietary) have elevated serotonergic activity (5-HT/5-HIAA ratio) in the hypothalamus, which results in a decreased stress response accompanied by a significant reduction in cortisol levels when exposed to social-mixing related stress [138, 186]. Tryptophan hydroxylase

2 (TPH2), rate-limiting enzyme of 5-HT synthesis in the brain, has been used as a therapeutic target for psychiatric disorder [187, 188]. The new strategy of psychotherapy aimed at restoring the normal gut microbiota and intestinal homeostasis via regulating the function of the gut-brain axis has been used in humans [184, 189, 190] and various animals including laying hens[151, 172].

## 7. Probiotics，*Bacillus subtilis*-Based Probiotics, Social Challenge-Induced Aggression.

### *7.1. Probiotics.*

Probiotics are commensal bacteria ("direct-fed microbials", DFM) that offer potential health beneficial bio-physiological effects to the host's stress response (acute, chronic or both). Probiotics aid animals in adapting to their environments and protect against pathogens by: 1) altering the microbiota profile with beneficial bacteria to prevent the growth of pathogens and to compete with enteric pathogens for the limited availability of nutrient and attachment sites; 2) producing bacteriocins (such as bacteriostatic and bactericidal substances) and short chain fatty acids against pathogens to regulate the activity and homeostasis of intestinal digestive enzymes and to increase mineral solubility; 3) reducing oxidative stress, inflammation, and acinar cell injury; 4) modulating host immune and inflammatory responses and restoring the intestinal barrier integrity which prevents pathogens from crossing the mucosal epithelium; 5) stimulating the endocrine system and attenuating stress-induced disorders of the HPA axis and/or SMA axis via the gut-brain axis; 6) inducing epithelial heat shock proteins to protect cells from oxidative damage; and 7) synthesis and secretion of neurotransmitter such as 5-HT and tryptophan [164, 191-193]. It has been stated in non-human primate models, the composition of the gut microbiota has potential effects on hosts' aggressive behaviors and anxiety symptoms [16, 25, 194, 195], which is similar to the findings reported in humans [196]. In rodent studies, germ-free (GF) animals with exaggerated HPA responses to social stress can be normalized by certain probiotics

[34, 197, 198]. In addition, probiotics have successfully attenuated anxiety and depressive behaviors in rat offspring separated from their mother [199, 200] and the obsessive-compulsive-like behaviors in house mice [201, 202]. These results support the psychobiotics theory [203] and provide a new insight into the possible use of probiotics to improve a host's cognitive function in humans and other animals including chickens [21, 204-206].

*7.2. Bacillus subtilis*

*Bacillus subtilis*, one of the three most common species of probiotic products in the U.S. [207], has been widely used as functional food supplements, such as in several dairy and non-dairy fermented foods, for improving human health [38, 208, 209]. Similarly, *Bacillus subtilis*-based probiotics have been used as antibiotic growth promoter alternatives in poultry [47, 210, 211]. *Bacillus subtilis* are spore-forming bacteria. They are heat stability, low pH-resistance (the gastric barrier), and tolerate multiple environmental stressors [212, 213]. Several mechanisms of the action of Bacillus spp. have been proposed: regulating intestinal microstructure [214] and digestive enzymes [215, 216]; synthesizing and releasing antimicrobial and antibiotic compounds [217]; increasing immunity [216, 218] and neurochemical activities including 5-HT [219-221] as well as affecting animal behavior [210, 222] followed various stressors. For example, in response to stimulations, *Bacillus subtilis* alleviate oxidative stress and improve mood status of hosts via the gut-brain axis [38, 223]. In addition, *Bacillus subtilis* can overproduce L-tryptophan [224-226], consequently increase 5-HT in the hypothalamus [225]. Tryptophan functions as an antidepressant and anti-anxiety agent [227-230] and eliminates nervous tension in mice [231, 232]. In our study, chickens were used as an animal model to assess if dietary supplementation of probiotic, *Bacillus subtilis*, reduces aggressive behaviors following social challenge.

Chickens, as social animals, show fear, depression, and anxiety in novel environments and show aggression towards other birds for establishing social dominance rank in unfamiliar social groups [233-235], which is similar to rodents used in human psychopharmacological studies [236-238]. The paired social ranking-associated behavioral test used in this study has been routinely performed in chicken behavioral analysis [224, 239-241]. The rationale and cellular mechanisms of the test is like the resident-intruder test which is a standardized method used in rodents for detecting social stress-induced aggression and violence [242, 243].

In the study, the role of probiotic, *bacillus subtilis*, on aggression in hens of DXL line was examined [244]. One-day-old female chicks with wing bands were reared in single-bird cages up to 24 weeks of age, then the hens with similar body weight were paired for the first aggression test (pre-treatment, day 0) in a novel floor pen. Behaviors were video-taped for 2 h immediately after released the two hens simultaneously into the floor pen to determine the dominant individual per pair. Following the behavioral test, the subordinate hens were continuously fed the regular diet, while the dominant hens were fed the diet mixed with 250 ppm probiotic (1.0 x 10^6 cfu/g of feed) for 2 weeks. The probiotic contains three proprietary strains of *Bacillus subtilis*. After the treatment (day 14), the second aggression test was conducted within the same pair of hens. The injurious behaviors were detected and analyzed as described previously (Table 1). The results indicated that, compared to their initial levels at day 0, the levels of threat kick were reduced (Figure 3a, $P$ = 0.04); the frequency of aggressive pecking tended to be lower (Figure 3b. $P$ = 0.053); and the levels of feather pecking was reduced but without statistic significant (Figure 3c. 58%. $P > 0.05$) in probiotic fed dominant hens. There was no change in the displaying of injurious behaviors in the regular diet fed subordinate hens between day 0 and day 14 (Figure 3 a-d). The behavioral changes in probiotic fed dominant hens were correlated with the changes of blood 5-

HT concentrations. Plasma 5-HT levels at day 14 were reduced toward the levels of controls (subordinates) in the probiotic fed dominant hens (Figure 4. $P = 0.02$) compared to their related levels prior to treatment (day 0). The similar correlations between aggressive behaviors and blood 5-HT concentrations have been identified in our previous genetic selection for prevention of social stress-induced feather pecking and aggression studies. Compared to MBB, KGB have lower blood 5-HT concentrations, as well as lower concentrations of blood DA and CORT and lower heterophil/lymphocyte (H/L) ratios (a stress marker), with lower frequency of injurious behaviors [3, 85] (Table 2). Bolhuis et al. [245] also reported that peripheral 5-HT activity reflects the predisposition to develop severe feather pecking in laying hens. A similar correlation between blood 5-HT levels and aggressiveness has also been detected in humans and various other animals; a lower level of blood 5-HT is associated with less aggressiveness in humans [246, 247] and canine [161]; while an elevated blood 5-HT level is determined in aggressive patients [248, 249] and teleost fish [250]. These results provide evidence for serotonergic mediation for aggressive behaviors and stress coping strategy; and chicken aggression can be reduced or inhibited by probiotic supplementation via directly or indirectly regulating of the serotonergic system.

Whether the changes of blood 5-HT levels in probiotic fed hens represent a similar change of 5-HT concentrations in the brain is unclear, as that 5-HT cannot pass the brain-blood-barrier and is regulated differently between brain neurons and peripheral tissues [251]. The plasma 5-HT is synthesized mainly by the enterochromaffin (EC) cells of the gut and stored in the platelets [252]. However, it has been proposed that platelet 5-HT uptake is a limited peripheral marker of brain serotonergic synaptosomes [161]. Dietary probiotic, *Lactobacillus plantarum strain* PS128, increases the levels of 5-HT as well as DA in the striatum, which is correlated with the improvement of anxiety-like behaviors in GF mice [253]. Similar results have been received from one of our current studies. In that study, chickens (broilers) were fed *Bacillus subtilis* from 1 day of age. At day 43, *Bacillus subtilis* fed chickens have higher levels of 5-HT in the raphe nuclei and lower levels of NE and DA in the hypothalamus than those of controls [56]. Probiotic fed chickens also have improved bone traits (bone mineral density, bone mineral content and robusticity index) via regulating the gut-brain-bone axis. In our heat stress (32 $^{o}$C for 10 hrs) study, *Bacillus subtilis* fed chickens have lower heat stress-related behaviors and inflammatory response in the hypothalamus compared to controls [210]. Taken together, these results evidence that probiotics, such as *Bacillus subtilis*, can affect hosts' brain neurotransmitter metabolism. However, further studies are needed to examine if there are correlations between the peripheral and brain 5-HT in probiotic fed chickens.

**8. Conclusion**

Enteric microbiota plays a critical role in hosts' response to acute and chronic stress. Social stress-induced changes of the gut microbiota lead to 'leaking out' of bacterial metabolites, affecting brain function, especially the function of the HPA axis and the SMA axis. These changes, consequently, negatively affect the physiological homeostasis, leading to mental disorders exhibiting abnormal behaviors including aggression. The current findings suggest that dietary inclusion of probiotics, such as the *Bacillus subtilis*-based probiotic, has positive effects on reducing agonistic behaviors in laying hens through modification of the serotonergic system. The novel approach could be transferred directly or indirectly to other species of farm animals that are subjected to painful husbandry procedures, such as dehorning of calves and teeth-clipping of piglets for preventing body injuries.

**Author Contributions:** H.W. C., S. J., and Y. J. H. designed this study. Y. J. H performed the experiments and analyzed the data. J. S. and H. W. C. wrote the manuscript. All authors have approved the final version of the manuscript.

**Funding:** This study was supported by the grant award (No: 2017-67015-26567) of the NIFA-AFRI, USDA (HWC). Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement of the USDA. The USDA is an equal opportunity provider and employer.

**Acknowledgments:** We thank the technicians of the USDA-ARS Livestock Behavior Research (West Lafayette, IN) for technical assistance.

**Conflict of Interests:** The authors have declared no conflict of interest.

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**Table 1.** Behavioral ethogram

| Behavior | Description |
|---|---|
| Feather Pecking: | One bird pecking at feathers of another bird, can be gentle (nibbling or gentle pecking in which feathers are not removed or pulled) or severe (vigorous pecking to feathers in which feathers are often pulled, broken, or removed). |
| Threat: | One bird standing with its neck erect and hackle feathers raised in front of another bird. |
| Aggressive pecking: | Forceful downward pecks directed at the head or neck of other birds |
| Threat Kick: | One bird forcefully extending one or both legs such that the foot strikes another bird. |

**Table 2.** Selection-induced alterations in blood concentrations of dopamine, serotonin, and corticosterone in laying hens

| Lines | Corticosterone (ng/mL) | Dopamine (ng/mL) | Epinephrine (ng/mL) | Serotonin (ng/mL) | H/L ratio[1] (x 100) |
|---|---|---|---|---|---|
| KGB[2] | 1.87 ± 0.19 | 0.59 + 0.08[a] | 0.30 + 0.06[a] | 11.8 + 0.07[a] | 13.0[a] |
| MBB | 1.49 ± 0.21 | 2.42 + 0.76[b] | 0.59 + 0.13[b] | 14.3 + 0.06[b] | 29.4[b] |

[a, b], Means within a column with different superscript are statistically different (n=12, $P < 0.05$).

[1], Heterophil/lymphocyte ratio

[2], The KGB and MBB lines were selected for high and low productivity and survivability resulting from cannibalism and flightiness. Respectively [254].

**FIGURES**

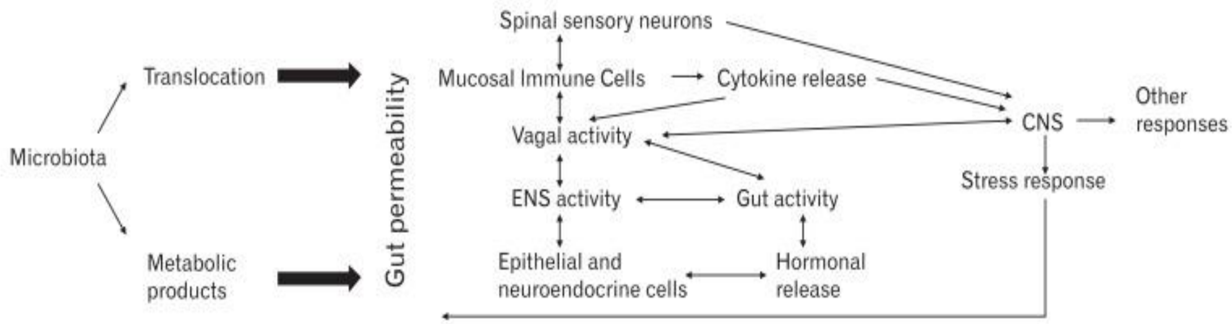


**Figure 1**. The microbiota-host interaction occurs at the level of the gastrointestinal mucosa via local neural, endocrine, and immune activities, influencing brain neurotransmitter expression, physiological homeostasis, and immunity. (Modified from Yarandi [255]).

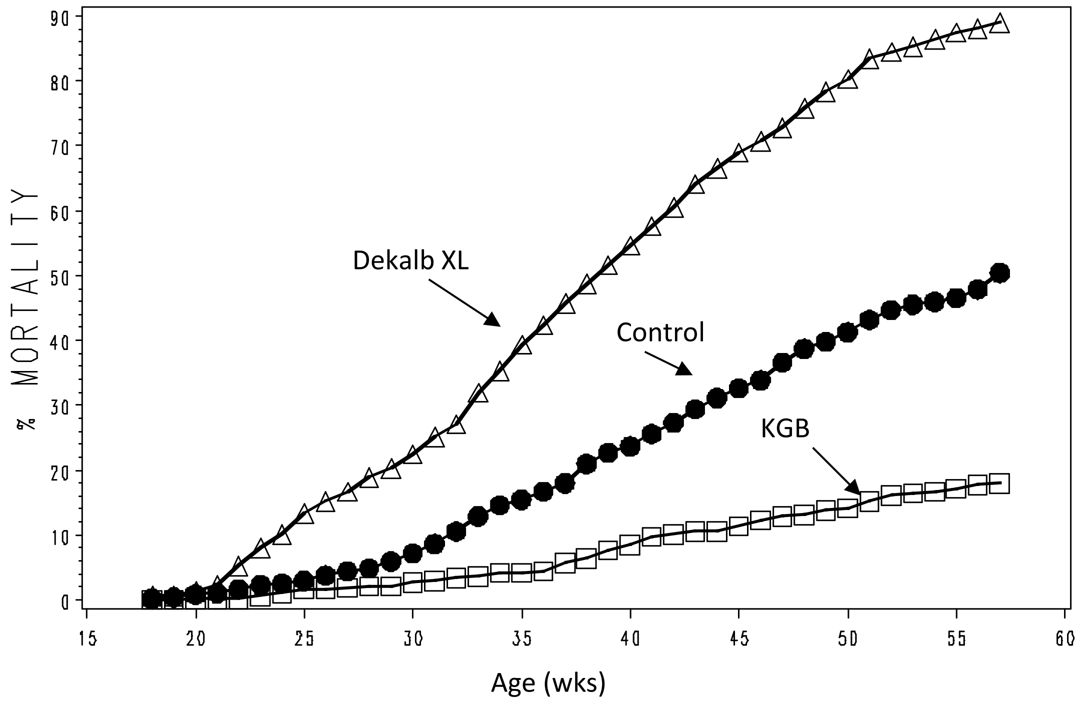


**Figure 2.** Annual percent mortality of the commercial (Dekalb XL), control, and KGB chickens. Notes, compared to birds from the Dekalb XL line and control line, selected KGB (kinder gentler bird) had the lowest mortality [254].

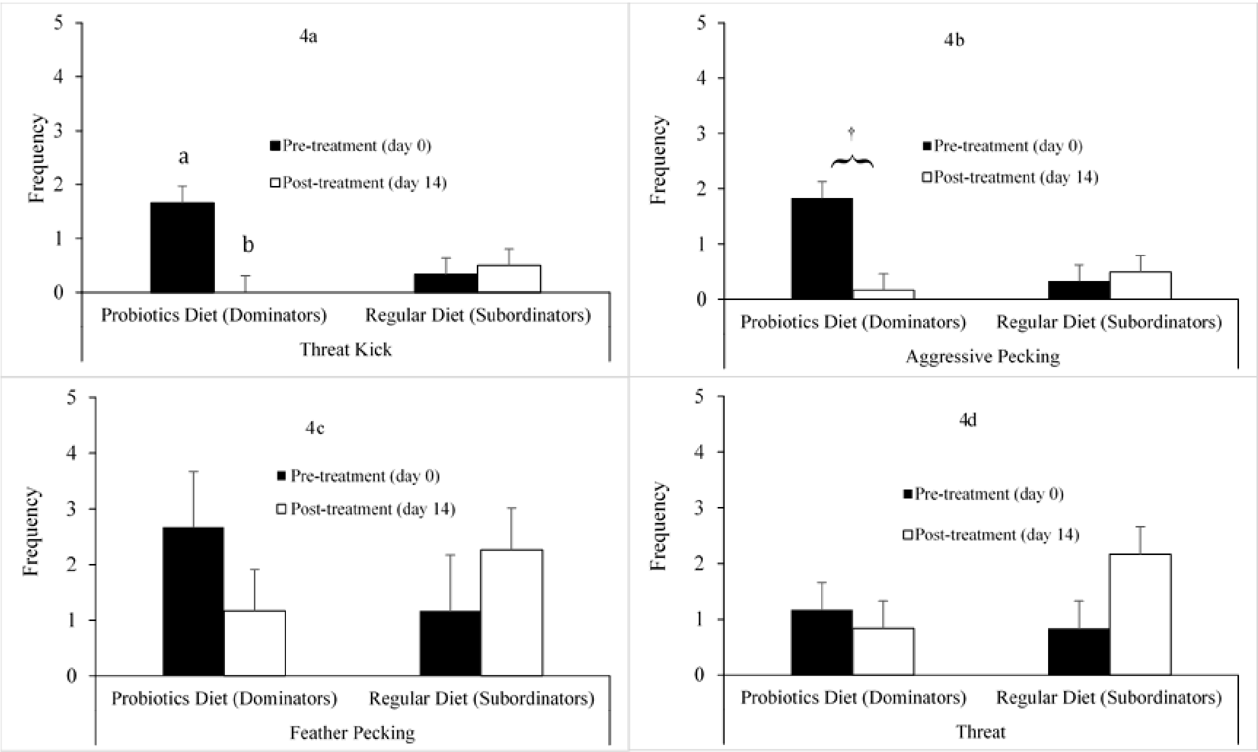


Figure 3. Frequency of aggressive behaviors at day 0 (pre-treatment) and day 14 (post-treatment) in probiotic fed hens and regular diet fed hens followed the paired social test. The exhibitions of aggressive behaviors in the regular diet fed subordinates were not affected by treatment ($P >$ 0.05, respectively), while the frequency of threat kick ($P = 0.04$) was reduced, aggressive pecking ($P = 0.053$) was tendency to be lower, and feather packing was declined (60%, $P = 0.33$) in probiotic fed dominates post-treatment. Notes: The treatment effects on the measured behaviors were reversed between dominants and subordinates during the 2nd social rank test.

[a,b] between the frequency at day 0 and day 14, least square means lacking common superscripts differ ($P < 0.05$); and †a trend difference ($0.05 \leq P < 0.10$).

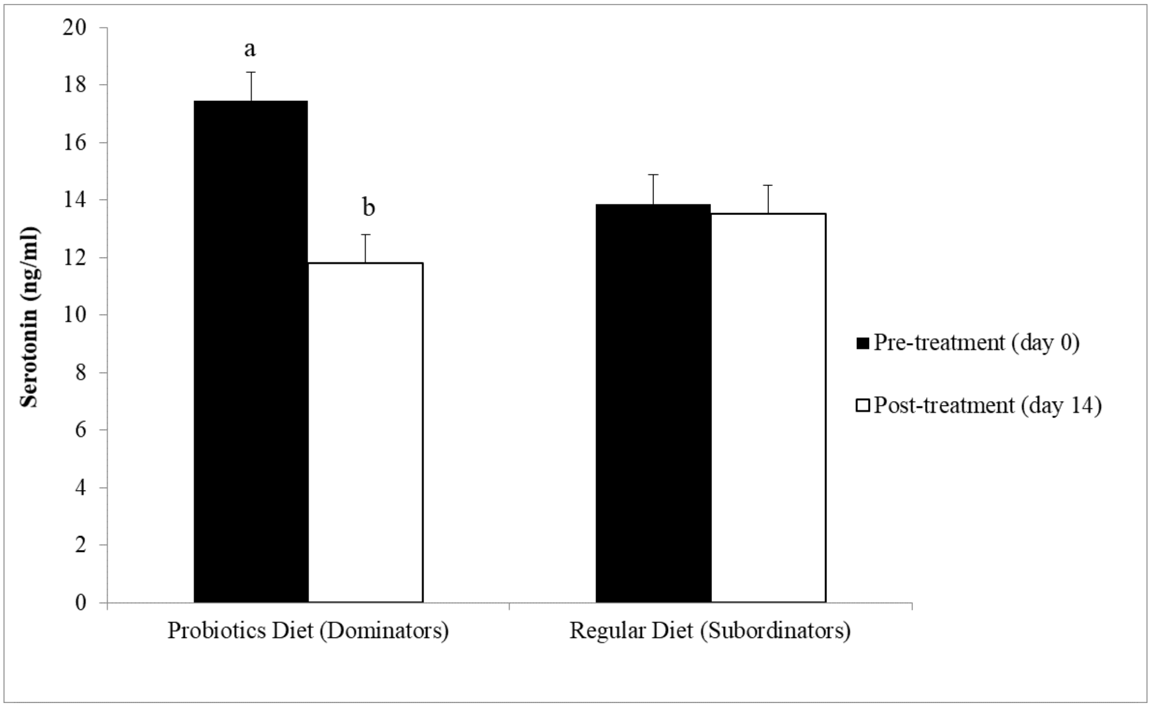


Figure 4. Plasma serotonin (5-HT) levels at day 0 (pre-treatment) and day 14 (post-treatment) in probiotic fed dominant hens and regular diet fed subordinate hens. Compared to subordinate hens, plasma 5-HT concentrations were higher in dominant hens at day 0 but without statistical different ($P = 0.24$); the difference was disappeared at day 14. Compared to the levels at day 0, blood concentrations of 5-HT were reduced in probiotic fed dominant hens at day 14 ($P = 0.02$) but were not in regular diet fed subordinate hens ($P > 0.05$).

[a,b] between the concentrations at day 0 and day 14, least square means lacking common superscripts differ ($P < 0.05$).