Chapter

Gut-Brain Axis: Probiotic, *Bacillus subtilis*, Prevents Aggression via the Modification of the Central Serotonergic System

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Abstract

Intestinal bacteria release various neuroactive compounds directly or indirectly regulating brain function to modulate host health and behavior through the gut-brain axis. Probiotics have been used as dietary supplements to target gut microbiota (microbiome) for prevention or therapeutic treatment of various diseases including mental disorders. 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 of an aggressive line were housed in single-hen cages. At 24 weeks of age, the hens were paired with similar body weight to identify the dominance rank (day 0). The subordinate and dominant of each pair were fed a regular layer diet or the diet mixed with 250 ppm probiotics for 2 weeks, then the second behavior test was performed between the same pair (day 14). The display of aggressive behaviors in the regular diet-fed chickens was not affected between the levels at day 0 and day 14, while the frequency of threat and aggressive pecking were reduced in the probiotic-fed chickens compared to the levels at day 0. These results suggest dietary probiotic, *Bacillus subtilis*, could be a suitable strategy for increasing hosts' mental health.

Keywords: probiotics, *Bacillus subtilis*, aggression, serotonergic system, gut-brain axis

1. Introduction

Human emotional susceptibility in an inter-group is associated with individual differences in the functions of the hormonal and/or neurochemical systems in response to internal and external stimulations. Aggression, as one of emotional disorders, has been commonly defined as feeling of anger or antipathy of an organism during social interaction, leading to hostile of violent behavior or attitude, provoking attack or confront toward another individual [1]. Aggression affects millions of people's health and welfare around the world annually, resulting in significant social destruction and economic costs.

Intestinal bacteria, as a virtual endocrine organ, release various neuroactive compounds directly or indirectly regulating brain function, including the serotonergic system, to regulate host health and behavior through the gut-brain

axis [2–10]. Intestinal bacteria, as well as *Bacillus subtilis*, used as probiotics are involved in tryptophan metabolism [11–14]. Tryptophan metabolites have roles in protecting intestinal mucosa from inflammation and regulating gut immune homeostasis [15–18]. Tryptophan, a precursor of 5-HT, directly affects brain 5-HT synthesis as that tryptophan can pass the brain-blood barrier [19, 20]. The brain serotonergic system plays a critical role in regulating behaviors, especially aggression [21, 22]. Concentrations of 5-HT and its metabolites, as well as the density of 5-HT receptors, have been used as major indicators of aggressive behaviors in humans and experimental animals [23–26].

Probiotics (also called psychobiotics or bio-friendly agents), defined "as a source of live (viable) naturally occurring microorganisms (direct-fed microbials, DFMs)", 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 in humans and various experimental animals [27–36]. Different probiotic strains, for example, have been investigated as functional food or therapeutic treatment of various diseases, including *Bifidobacterium bifidum* [37–39]; *Bifidobacterium bifidum* (BGN4) and *Bifidobacterium longum* (BORI) [40, 41]; *Bifidobacterium pseudocatenulatum* [42]; *Lactobacillus helveticus* [31, 32]; *Lactobacillus plantarum* [43]; *Lactobacillus paracasei* (KW3110, [44]); *Lactobacillus rhamnosus* [45]; and *Clostridium butyricum* [46]. The results collected from these studies 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 [47, 48].

Bacillus subtilis, as one of the three most common species of probiotic products in the United States [49], has been widely used as functional feed supplements, such as in a number of dairy and non-dairy fermented foods, for improving human health and well-being [50–52]. Similarly, *Bacillus subtilis*, as growth promoters, has been demonstrated to improve chickens' growth performance [53–55]; regulate intestinal microstructure [56] and digestive enzymes [57, 58]; synthesize and release antimicrobial and antibiotic compounds [15, 59–63]; increase immunity [57, 64] and neurochemical activities including serotonin [8, 65, 66] as well as affect animal behavior [67, 68] following various stressors. In addition, *Bacillus subtilis* can overproduce L-tryptophan [11, 69], consequently increase 5-HT in the hypothalamus [70], function as an antidepressant and anti-anxiety agent [71, 72], and eliminate nervous tension in mice [73]. In the current study, chickens were used as an animal model to assess if dietary supplementation of probiotic, *Bacillus subtilis,* reduces aggressive behaviors following social challenge.

2. Gut microbiota and the gut-brain axis

Gut microbiota is a collective name of ten of trillions of microorganisms living in our intestine, including more than 35,000 different species of known bacteria [74]. Microbiome refers to the collective genomes of more than 3 million genes of the microorganisms in a particular environment. Gut microbiota, like a virtual endocrine organ, reacts to various internal and external stimuli [5, 75–78]. Consequently, gut microbiota influences and regulates hosts' health and mood statue including aggression by integrating metabolic, immune, endocrine, and neural reactions through the bidirectional communication of the gut-brain axis (**Figure 1**) [7, 33, 79–83]. Gut microbiota in hosts, for example, regulates brain neurotransmitters [84–87] such as serotonin (5-HT) through releasing its precursor, tryptophan, an essential amino acid [75, 88–90]. Serotonin is a key neurotransmitter within the brain, contributing to the development of the central nervous system (CNS)

[91–94] and social behavior [95, 96], affecting mental health [97–100] and providing therapeutic strategy for treating or preventing stress reaction and related neuropsychiatric disorders [94, 97].

2.1 Social stress and the hypothalamic-pituitary-adrenal axis

The social world is filled with different types of interactions, such that social challenges (fight-or-flight) and social environmental changes (group instability), as stressors, can cause highly potent stress [101–103]. Some management practices used in the intensive farm animal production systems, for example, may cause stress in animals. Farm animals are often housed in large groups at high densities, and during the animals' lifetimes, they are repeatedly transported to new environments and mixed with unfamiliar individuals. Based on the social network theory, animal group disruption can lead to social stress and related aggression [104] when they are unable to cope with these management practices and/or to reset their dominance rank (a form of animal social structure hierarchy) [105, 106].

Aggression has been commonly defined as feeling of anger or antipathy during social interactions, leading to hostile or destructive behaviors or attitudes, provoking physical or verbal attack or confront toward another individual [1]. Aggressive encounters among conspecifics for obtaining or maintaining a socially dominant position or rank cause a negative or unstable social environment to activate the hypothalamic-pituitary-adrenal (HPA) axis which is known as the key stress response system [107–109]. Cortisol (or corticosterone, CORT, in rodents and birds), as one of the final hormones released from the adrenal cortex, has multifunctional functions in both normal and abnormal states, regulating behavioral styles, metabolic patterns, and endocrine and immune functions, and ensuring an adequate coping strategy and well-being [110–112]. In mammals, hypercortisolism in response to novel environment exposures has been evidenced to be of adrenal origin [113, 114]. The value of circulating cortisol (or CORT) has been used as a criterion of stress response in humans and various animals [115–117]. Corticotrophin-releasing factor facilitates aggression [118, 119], and related elevation of CORT (or cortisol) also affects neuroendocrine functions through binding to their receptors [120, 121], causing neuron loss in the hippocampus [122], dysfunction of the serotonergic system [123], and inhibition of immunity (increased pro-inflammatory cytokines as neuroinflammatory response) within stress-sensitive brain regions [124]. These changes finally lead to the development of psychological disorders such as aggression [109, 125]. Similar to that the HPA hyperactivity generates aggressive behavior, exogenous glucocorticoid treatments increase exacerbated aggressiveness in both humans and experimental animals [123, 126]. Taken together, emerging

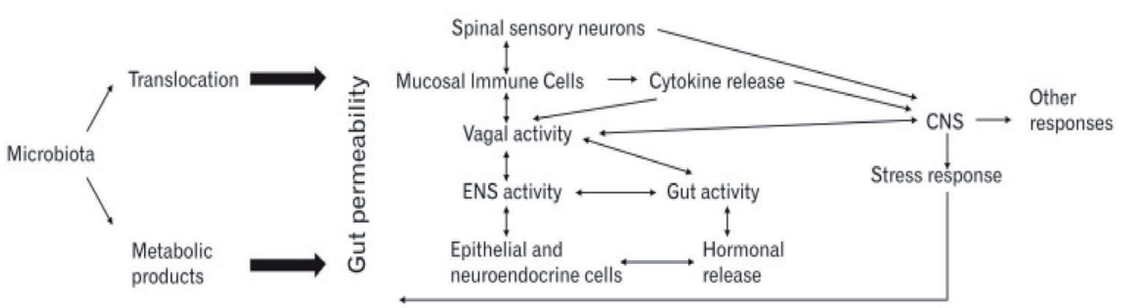

Figure 1.
The microbiota-host interaction occurs at the level of the gastrointestinal mucosa via local neural, endocrine, and immune activities, influencing brain neurotransmitter expression, physiological homeostasis, and immunity (modified from [79]).

evidence have indicated that the changes of the programming of HPA stress reactivity [127] cause long-term effects on the host physiological homeostasis and neurobehavioral functions [128].

2.2 Microbiota, stress-associated aggression

Aggression within a group is to establish a dominance hierarchy when the animals are first brought together in a common environment [129–131]. From an evolutionary perspective, aggression is adaptive behaviors that are related to an individual's survival, growth, and reproductive success within a group [132, 133]. However, aggression with long-term impact is often destructive and maladaptive in today's society, affecting millions of people's health and well-being around the world annually, resulting in significant social destruction and economic costs [134–136].

Intestinal microbiota plays an important role in regulation of social behavior, emotional expression, and mental health within the animal kingdom [3, 137, 138]. They are essential players in stabilizing homeostasis of the GI tract in response to both acute and chronic stress via the microbiota-gut-brain axis [16, 139]. Normally, intestinal microbiota provides protection for animals by competing for attachment sites and nutrients with pathogens as well as production of antimicrobial peptides and neuroactive compounds [140, 141]. The gut microbiota is also a key pathway to modulate brain processing the integrated information received from 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) [5, 137]. Under social challenges, stress and related oxidative damage cause anatomical and functional disorders of the GI tract by: (1) disrupting the commensal bacterial populations and colonization, thus reducing beneficial bacteria and increasing pathogens; (2) increasing pathogen survivability and innovating capability; (3) disrupting absorption of nutrients and minerals, including calcium; (4) disrupting microbial neuroendocrine functions; (5) disrupting the gut epithelial barrier, thereby increasing intestinal permeability causing the gut to leak certain bacteria (leaky gut), resulting in metabolic disorder; (6) damaging epithelial cells, thus producing free radicals and reducing antioxidant efficacy; and (7) interrupting intestinal integrity, thereby leading to intestinal inflammation [142–144]. These changes in gut microenvironment affect brain functions, resulting in exacerbated HPA axis activity, increased chronic inflammation, and/or disrupted neurotransmitter balance, leading to emotional damage [139, 145] and mental disorders [137, 138]. Sudo et al. [146] reported a correlation between the changes of gut microbiota and the function of the HPA axis. Germ-free (GF, antibiotic-treated microbiota-deficient or raised without any exposure to microorganisms) rats show exaggerated HPA responses to psychological stress [75, 147] with significantly higher levels of both ACTH and CORT in response to restraint stress compared to control rats [146]. In GF mice, gut microbiota also modulates the 5-HT synthesis and release at both the brain and peripheral levels directly and indirectly via the microbiota-host interactions [148]. Reduced 5-HT activity (a 5-HT deficiency) has been associated with personality traits (interspecific social behavior), such as impulsivity and aggression, and deteriorated stress coping capability (increased stress response) in humans and various animals [149, 150] including chickens [24, 151–154].

3. Serotonin and aggression

Serotonin, as an ancient chemical, is a key neurotransmitter. It plays a critical role in shaping social responses by regulating both basic (proactive) behaviors

(such as feeding, drinking, and sexuality) and reactive behaviors (fearfulness, anxiety, and cognition) including aggressive behaviors [155, 156] and mood disorders [157, 158]. Abnormalities of blood and brain levels of 5-HT, 5-HIAA (its metabolite, 5-hydroxyindoleacetic acid), tryptophan, and its receptors have been used as major indicators or targets in the diagnoses and treatments of psychiatric and compulsive disorders in humans and various experimental animals [62, 63, 159, 160].

In the CNS, 5-HT functions to inhibit aggression, thereby controlling domestic behaviors [161–163]. The 5-HT deficiency theory of aggression is driven from the negative correlation between the changes of the CNS 5-HT and aggressiveness in humans [164, 165], non-human primates [166], rodents [25, 167], and chickens [24, 168]. Aggressive animals have low levels of 5-HT in the brain, including in the hypothalamus [169–171]. Experimental increase of 5-HT and/or 5-HIAA in the brain, such as in the lateral hypothalamus and amygdala, blocks or retracts killing behavior in rodents [172, 173]. Hypothalamic injection of a 5-HT1a agonist inhibits aggression in male hamsters [174]. Depletion of brain 5-HT in TPH2 mutant mice marks aggression and lowers habituation in novel environments [149]. In addition, 5-HT1BR knockout mice show increased aggression and impulsivity [175, 176]. The implication of 5-HT successfully relieves the depression syndromes in humans [177] and reduces aggressive behaviors in primates and rodents [178, 179].

In the peripheral system, however, pathophysiological roles of 5-HT in behavioral and motivational regulations are unclear. Reduced, elevated, and unchanged blood 5-HT concentrations have all been reported in association with behavioral dysfunctions, including aggressiveness [180, 181]. The conflicting data from different investigations could be related to the differences in species of animals, behavioral evaluations, and/or stressors used as well as duration and frequency of stressors presented.

4. Chicken as an animal model for social stress and related aggression

Human 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 [182, 183]. Various social and biological factors are associated with the development and expression of aggressive behaviors, including environmental, genetic, cognitive, hormonal, and neurotransmitter circumstances [184]. Aggression and associated mental illness are an emerging public health problem [29]. Animal models are critical for investigating the potential biological processes involved in human aggression and mental disorders.

Although there are dissimilarities between humans and chickens, as indicated, the neural circuitry for aggression and social behavior appear to be evolutionarily conserved across the vertebrates [185]; chickens have been used as an animal model in various clinical and psychopharmacological studies, such as anxiety, depression, and aggression [186, 187]. A commercial hen can have more than 300 offspring with similar genetic characteristics during her lifetime, and chicks can be hatched without maternal condition effects. Previous studies have reported that birds' brain possess a core "social behavioral network" which is humongous to the social behavioral network of mammals [188]. There are evidences that the central nuclei involved in moodiness in avian, at least in part, are morphofunctional homologous to the mammalian nuclei [189], such as the hypothalamus [190], nucleus taeniae (homolog to the amygdala of mammals, [191, 192]), and Raphe nucleus [193]. These nuclei exert similar cognitive abilities and consciousness [194] with capability of plasticity in response to environmental stimulations [195]. In addition, there are similar distributions of neurotransmitter receptors, including serotonergic receptors

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 1.
Behavioral ethogram.

between birds and mammals [196, 197]. Particularly, it has been evidenced that the development and function of avian HPA axis in response to stimuli are analogous to those of mammals [198, 199] and there are similar distributions of cortisol-like molecules in the same organs from both birds and mammals [200]. Birds' CORT biosynthesis and metabolism can be stimulated in the same way as that of mammals [201–203]. During breeding, functional integrations among behavior, physiology, morphology, and environment in chickens may create suites of various traits that are simultaneously acted upon each selection program [204]. For example, selection based on individual production capability (phenotypic characteristics) increases animals' competition and aggression [205, 206] such as potentially injurious feather pecking and cannibalism. For instance, through more than 20 years of selection, egg production had been increased significantly in a former commercial Dekalb XL strain, whereas mortality due to aggression and cannibalism in non-beak trimmed hens had also increased about 10-fold [132, 152].

Results from previous studies conducted in our laboratory have found that chicken strain differs in social reactions through a diversely genetic selection program. The DXL hens have distinct stress levels in attempting to adapt to their social environments [207, 208] and exhibit different levels of aggressiveness [168]. In addition, neurotransmitters, 5-HT and dopamine (DA), and the hypothalamic-pituitary-adrenal (HPA) axis are regulated differently between chicken strains [168, 207]. There results suggest that injurious behaviors and related social sensitivity of chickens, similar to that in mammals, are regulated via the serotonergic system and the HPA axis [123, 209], and mechanisms underlying aggression in laying hens may be analogous to those of humans and rodents [210]. Similarly in humans and rodents, stress-induced destruction of intestinal bacteria disturbs the bilateral connection of the microbiota-gut-brain axis in chickens, affecting physiological homeostasis and behavioral exhibition [211]. In one of our studies, the role of probiotic, *Bacillus subtilis*, on aggression in DXL line was examined. In the study, one-day-old female chicks were reared in single-bird cages up to 24 weeks. At 24 weeks of age, the hens with similar body weight were paired for the first aggression test (pre-treatment, day 0) in a novel floor pen [212]. Behaviors were video-taped for 2 h immediately after releasing 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 (1.0×10^6 cfu/g of feed) for 2 weeks. The probiotic contains three proprietary strains of *B. subtilis.* After the treatment (day 14), the second aggression test was conducted within the same pair of hens. The video recording was analyzed for frequency of feather pecking, threat, aggressive pecking, and threat kick by the routine lab procedures (**Table 1**) [154, 213].

5. Probiotic, *Bacillus subtilis*, and aggression

As social animals, chickens show fear, depression, and/or anxiety in novel environments and show aggression toward ones for establishing social dominance rank in unfamiliar social groups [214, 215], which is similar to rodents used in human psychopharmacological studies [216, 217]. The paired social ranking-associated behavioral test used in this study has been routinely performed in chicken behavioral analysis [154, 213, 218]. The rationale and cellular mechanisms of the test is similar to the resident-intruder test which is a standardized method used in rodents for detecting social stress-induced aggression and violence [219, 220].

5.1 Probiotics and *Bacillus* spp.

A healthy intestinal microbiota community is important for maintaining physiological and behavioral homeostasis as that the gut microbiota regulates appetite, local and systemic immunity, stress responses of the HPA and sympathetic-medullary-adrenal (SMA) axes, and circadian rhythms [5, 221]. The new strategies of psychotherapy aimed at restoring the normal gut microbiota and intestinal homeostasis have been developed for the prevention and/or reduction of stress-induced abnormal behaviors and mental disorders.

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 of intestinal digestive enzymes and energy homeostasis 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 and/or SMA axes via the gut-brain axis; and (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 [16, 17, 222–226]. In both human and rodent studies, probiotics reduce cognitive dysfunction, decrease the stress response and related oxidative damage by lowing plasma CORT and ACTH levels, restore hippocampal 5-HT levels, and normalize immunity with low plasma levels of TNF-α (tumor necrosis factor-α), a proinflammatory cytokine, but high levels of IL-10 (Interleukin-10), an anti-inflammatory cytokine [67, 227–230]. It has been stated in nonhuman primate models that the composition of the gut microbiota has potential effects on hosts' aggressive behaviors and anxiety symptoms [127], which is similar to the findings reported in humans [137, 231–233]. In rodent studies, GF animals with exaggerated HPA responses to social stress can be normalized by certain probiotics [147]. In addition, probiotics have successfully attenuated anxiety and depressive behaviors in rat offspring separated from their mother [234–236] and the obsessive-compulsive-like behaviors in house mice [28, 237]. These results support the psychobiotics theory [238] and provide a new insight into the possible use of probiotics to improve a host's cognitive function in humans [9, 40, 41, 239–243].

A proposed strategy for improving human health is dietary supplement with probiotic microorganisms including Bacillus species [52, 244]. *Bacillus subtilis* is spore-forming bacteria. They have heat stability and low pH-resistance (the

gastric barrier), and tolerate multiple environmental stressors [245, 246]. Several Bacillus spp. such as *B. coagulans* and *B. subtilis* have been used as probiotics in both humans [247–249] and animals [246] including poultry [65, 67, 250, 251]. Several mechanisms of action of *Bacillus* spp. have been proposed: the improvement of hosts' growth, survival, and health status via their anti-inflammatory functions through immunomodulation and cytoprotection [252, 253]. And, they have been used as antibiotic growth promoters alternatives with health-promoting benefits by naturally synthesizing proteins, enzymes, antimicrobial peptides, vitamins, gut flora modulation to promote beneficial microbiota along the GI tract and to correct and repair immunological and gut morphological alterations [244, 246, 250, 254, 255]. In addition, numerous studies have shown that probiotic-induced changes in the composition of gut microbiota lead to alterations of neuroendocrine functions. For example, in response stimulations, *B. subtilis* alleviate oxidative stress, provoke a specific biological response, and improve mood status of hosts via the gut-brain axis [52, 147, 256].

5.2 *Bacillus subtilis*-based probiotic and social challenge-induced aggression

Aggression, in nature, is associated with competition (natural selection) for survival and reproduction [22, 257, 258] (please also see above for the detailed description). However, in artificial production environments, such as in the poultry industry, aggression causes increased social stress and feather and body damage, in some instances these injuries leading ultimately to cannibalism. Cannibalism is a major concern related to non-beak trimmed bird deaths in current housing environments [259, 260]. Beak trimming (BT) is a routine procedure practiced in the US egg industry for reducing social stress by preventing and/or inhibiting feather pecking and cannibalism. However, BT is criticized for causing tissue damage and pain (acute, chronic, or both), negatively affecting the welfare of billions of chickens annually [261, 262]. Considerable concern from the public has led to a growing movement against procedures causing pain and suffering in farm animals. In response to growing pressures, housing environments of laying hens have been modified and/or various dietary supplementations have been provided in attempts to prevent social stress and stress-associated injurious behaviors. For instance, modifications include reducing light intensity, changing the nutritive value or taste of diets [263–265], providing straw or grain [266, 267] or pelleted diets [263], housing hens in floor-pens [268], and developing enriched cages [269, 270]. However, these methods have had limited success and provide no guarantee of preventing these injurious behaviors. Therefore, an obvious solution is to develop a welfare-friendly alternative to BT that minimizes social stress, thereby preventing feather pecking and cannibalism. The hypothesis was tested in this study: probiotics could be an alternative to beak trimming in chickens for reducing feather pecking and aggression via regulating the gut-brain axis.

Based on the 5-HT deficiency theory of aggression, social challenge-induced changes in 5-HT concentration were examined in this study. In the current study, the data showed that prior to the treatment (day 0), plasma 5-HT levels were higher (26% increase) in the dominant hens than that of subordinate hens but were not statistically significant (P = 0.24. $\text{Dominant}_{5\text{-HT}}$ = 17.46 ng/ml, $\text{Subordinate}_{5\text{-HT}}$ = 13.87 ng/ml). This finding is in agreement with the results reported previously [152, 168]. In those studies, higher plasma levels of 5-HT were detected in hens from mean bad bird (MBB) strain, a high aggressive strain selected for both low productivity and low longevity resulting from injurious pecking and cannibalism, compared to hens from kind gentle bird (KGB), a low aggressive strain selected for both high productivity and high longevity. In

addition, Bolhuis et al. [271] proposed that blood 5-HT activity is correlated with the development of 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, that is, a lower blood 5-HT level was associated with less aggressive individuals in humans [180, 272] and canine [273], while an elevated blood 5-HT level was determined in patients with aggressive behaviors [274] and in aggressive teleost fish [275].

Post-treatment (day 14), plasma 5-HT levels were reduced toward the levels of controls (subordinates) in the probiotic fed dominant hens (P = 0.02) compared to their related levels prior to treatment (day 0) (**Figure 2**). There were no treatment effects on plasma 5-HT concentrations in subordinate hens fed a regular diet (P = 0.88). Although the reasons of the reduction of plasma 5-HT concentrations in probiotic fed hens are still unclear but could be similar to those proposed in probiotic-treated patients with intestinal inflammatory disorders such as irritable bowel syndrome (IBS) and IBS experimental animals. Probiotics reduce IBS-associated abdominal pain and abnormal bowel habits [276, 277] through regulation of both the central and peripheral serotonergic systems via the microbiota-gut-brain axis [278, 279] and gut epithelial enterochromaffin cells [19]. In the peripheral system, probiotics reduce or inhibit IBS-associated serotonergic system abnormalities, that is, great hypersensitivity and spontaneous release of 5-HT [280, 281]. Serotonin reuptake transporter (SERT or 5-HTT) has functions in inactivating 5-HT. Down-regulation of SERT receptors has been found in the intestinal mucosa of IBS patient whose symptoms are similar to those found in the SERT knockout mice [282]. Serotonin 5-HT3 receptor antagonist also has shown efficacy in treating IBS patients [278, 283]. In addition, Wikoff et al. [284] reported that conventional mice had lower concentrations of 5-HT compared to GF mice. GF mice also had an exaggerated stress response [146] with anxiety-like behaviors [285]. These abnormal behaviors in GF mice can be inhibited or reduced by feeding probiotics [286] or transplanting fecal samples of conventional mice [287]. The current and previous

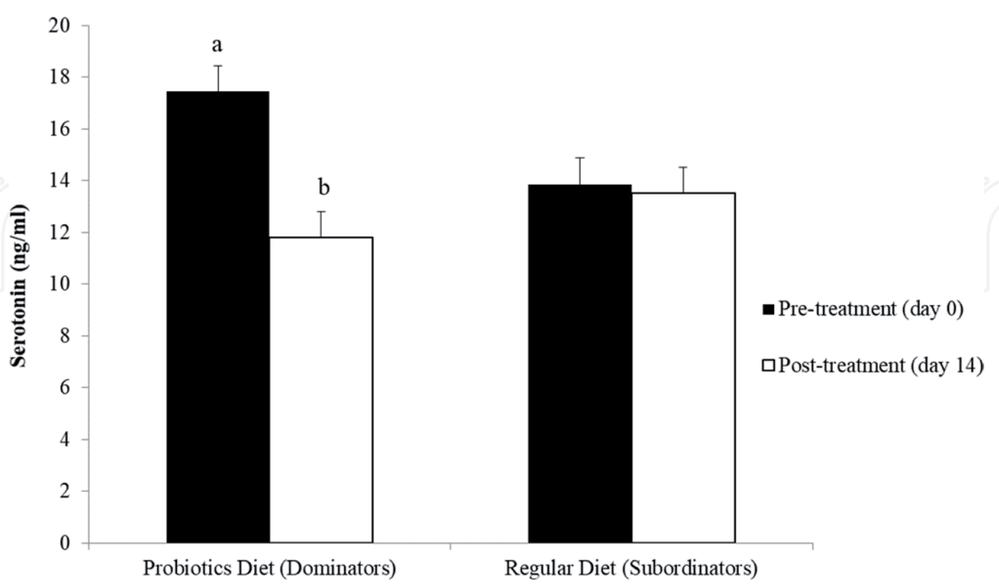

Figure 2.
Plasma serotonin (5-HT) levels at day 0 (pre-treatment) and day 14 (post-treatment) in probiotic fed dominant hens and regular diet fed subordinate hens. Compared to subordinate hens, plasma 5-HT concentrations were higher in dominant hens at day 0 but without statistical difference (P = 0.24); the difference 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).

results indicate that normal health gut microbiota plays an important role in regulating social stress and stress-associated behaviors.

Whether the changes of blood 5-HT levels in probiotic fed dominant hens represent a similar change of 5-HT concentrations in the brain is unclear, as 5-HT cannot pass the brain-blood barrier and is regulated differently between brain neurons and peripheral tissues [288]. The plasma 5-HT is synthesized mainly by the EC cells of the gut and stored in the platelets [289]. However, it has been proposed that platelet 5-HT uptake is a peripheral marker of brain 5-HT [273]. Dietary probiotic, *Lactobacillus plantarum strain* PS128, increases the levels of 5-HT as well as dopamine in the striatum, which is correlated with the improvement of anxiety-like behaviors in GF mice [290]. Similar results have been received from our current studies. In one study, chickens (broilers) were fed *Bacillus subtilis* from day one. At day 43, *Bacillus subtilis* fed chickens had higher levels of 5-HT in the raphe nuclei and lower levels of norepinephrine and DA in the hypothalamus than controls [291]. Probiotic fed chickens also had improved bone traits (bone mineral density, bone mineral content, and robusticity index). Under heat stress (32°C), *Bacillus subtilis* fed chickens had lower heat stress-related behaviors and inflammatory response and reduced IL-6 levels in the hypothalamus compared to controls [67]. Further studies, however, are needed to examine if there are correlations between the regulations of peripheral 5-HT and CNS 5-HT in probiotic fed hens.

The gut commensal microflora may have an indirect effect on 5-HT synthesis by regulating tryptophan metabolism. The degradation of tryptophan, a precursor of 5-HT, is mainly through the kynurenine pathway which regulates over 95% of tryptophan in the peripheral system and is functionally mediated by gut microbiota and probiotics [147, 292]. In the present study, the tryptophan level was not significantly affected by dietary probiotic ($P = 0.35$), but the initial levels of tryptophan in dominant hens were approximately 28% higher than the subdominant hens ($P = 0.21$) (**Figure 3**). The pattern of changes in blood concentrations of tryptophan in probiotic fed dominant hens was correlated with the changes of peripheral concentrations of 5-HT, indicating that probiotic may directly or indirectly regulate 5-HT synthesis in the peripheral system.

In this study, behavioral changes in dominant hens were correlated with the changes of blood 5-HT concentrations following fed probiotic. In the probiotic fed dominant hens, the levels of threat kick were reduced (**Figure 4a**, $P = 0.04$), and the frequency of aggressive pecking tended to be lower (**Figure 4b**, $P = 0.053$). The levels of feather pecking in dominant hens were reduced compared to their initial levels at day 0 but without statistic significant (**Figure 4c**, 58%, $P > 0.05$). There was no change in injurious behaviors in the regular diet fed subordinate hens between day 0 and day 14 (**Figure 4a–d**). The same or similar cellular mechanisms proposed in humans and rodents may be applied to the changes in the probiotic fed dominant hens. In humans and rodents, probiotics directly manipulate commensal bacteria releasing neuroactive factors, such as 5-HT and norepinephrine [8], and indirectly affect the neurotransmitter metabolisms, such as tryptophan as well as cytokines, through the microbiota-gut-brain axis [5, 40, 41, 279, 293–297]. In addition, the effects of probiotics, including *B. sublitis*, on behavioral exhibitions have been conducted on GF mice. Bercik et al. [287] reported that anxiety behaviors can be induced in less anxious phenotypic mice by colonization of the gut bacteria from anxiety-like phenotypic mice (FMT, fecal microbiota transplant). Probiotics also have therapeutic effects on neurodevelopmental disorders [28, 95, 298–301], for example, reduced anxiety-like behaviors by providing *L. helveticus*, *Mycobacterium vaccae*, and/or *Bifidobacterium* strains [231, 287, 302, 303], and alleviated autism-related stereotypic behaviors by treating with *Bacteroides fragilis* [304] and behavioral dysfunction with *Lactobacillus reuteri* [301, 305].

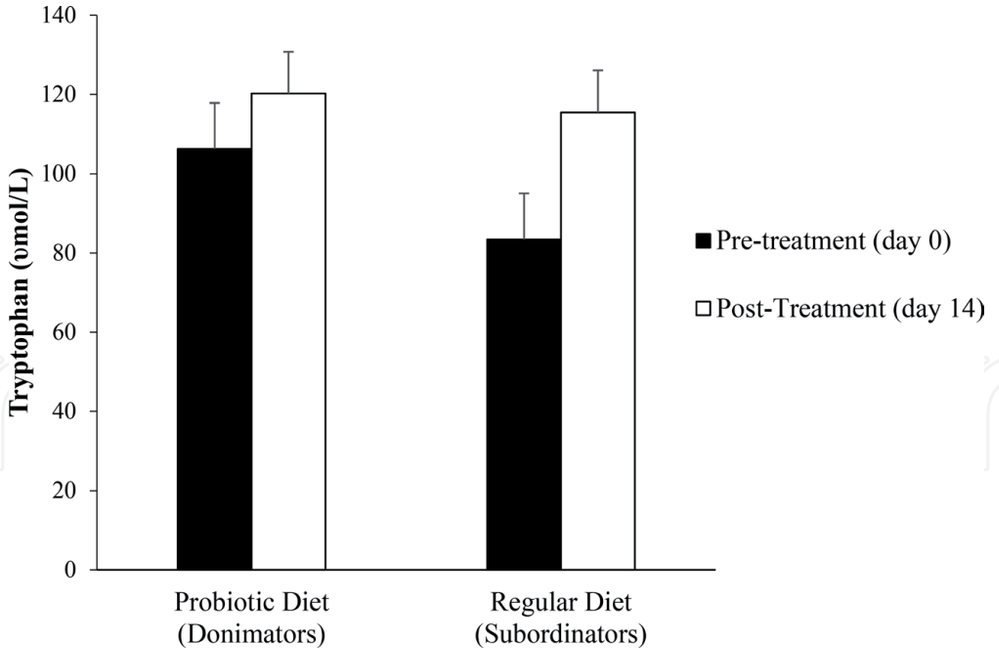

Figure 3.
Plasma tryptophan levels at day 0 (pre-treatment) and day 14 (post-treatment) in probiotic fed hens and regular diet fed hens. Prior to treatment, dominant hens had higher tryptophan concentrations compared to subordinates but the difference did not reach statistical difference (P = 0.21). There were no treatment effects on tryptophan concentrations in both probiotic fed hens and regular diet fed hens (P > 0.05, respectively).

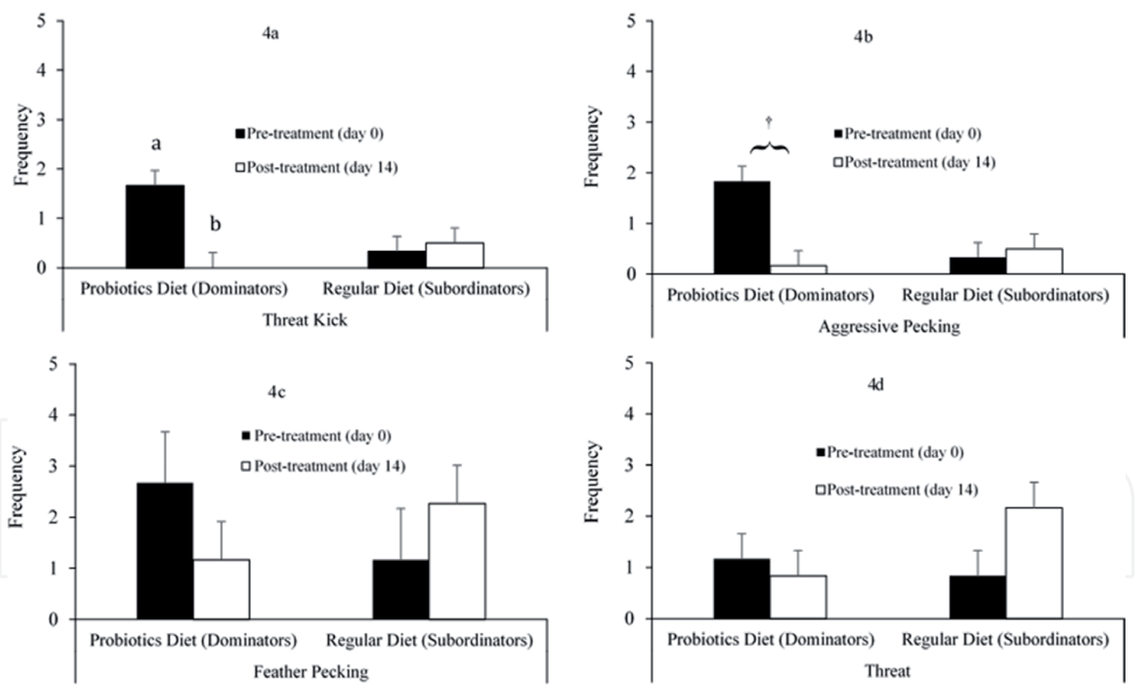

Figure 4.
Frequency of aggressive behaviors at day 0 (pre-treatment) and day 14 (post-treatment) in probiotic fed hens and regular diet fed hens followed the paired social test. The exhibitions of aggressive behaviors in the regular diet fed subordinates were not affected by treatment (P > 0.05, respectively), while the frequency of threat kick (P = 0.04) was reduced, aggressive pecking (P = 0.053) was tend 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 ≤ P < 0.10).

In the current study, body weight gain and hen-day egg production were not affected in the probiotic fed hens compared with the regular diet fed control hens (Control = 2.83%, Probiotics = 2.2%, *P* = 0.76; Control = 73.6%, Probiotics = 87.5%,

P = 0.18, respectively). Previous studies have reported the beneficial effects of dietary supplementation of probiotics on daily weight gain, finished body weight, and feed conversion rate in broiler chickens [306, 307], turkeys [308], and swine [309]. Several studies also reported that probiotic diets improved egg production in hens [310]. The underlying mechanisms of these effects may be related to the beneficial bacterial growth in the gastrointestinal tract to facilitate the fermentation process which improves the digestion and utilization of nutrients in animals [311]. The beneficial effects on growth performance, however, are affected by the bacterial strains, preparation process, dosage, animal's age, and genetic type [312, 313]. In the current study, the probiotic was provided for 2 weeks only, which may not be sufficient to functionally improve both growth and production performance.

6. Conclusions

Our data suggest that dietary inclusion of probiotics has positive effects on reducing agonistic behaviors in laying hens through modification of the serotonergic system without negative effects on growth and production performance. The data indicate that dietary probiotic supplementation could be a useful management tool for preventing aggressive behaviors in laying hens. In addition, the current chicken strain could be a useful model to investigate mechanisms underlying the potentially probiotic therapy for preventing and reducing emotional susceptibility associated with psychiatric disorders such as depression and anxiety in humans.

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