Probiotic *Bacillus subtilis* on Laying Hens

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Bacillus subtilisis one of the three most common species of probiotic products in the U.S. and has been used widely as a functional feed supplement such as in several dairy and non-dairy fermented foods for improving human health and well-being. Similarly, *Bacillus subtilis*-based probiotics have been used as antibiotic growth promoter alternatives in poultry. *Bacillus subtilis* are spore-forming bacteria. They are heat stable, low pH-resistant (the gastric barrier), and tolerate multiple environmental stressors.

Keywords: chicken ; social stress ; serotonin ; probiotic

1. Production Environments and Related Stress in Commercial Laying Hens

Chickens as well as other farm animals are constantly selected by both nature (natural selection) and humans (artificial selection). During selection, the animals' biological and behavioral characteristics have been constantly changed [1][2]. The process is affected by multiple factors including their surrounding environments, by which the animals have been selected for increased fitness (that is survival and reproductive success) over generations.

Commercial chickens have been selected for production (laying hens for eggs and broilers for meat) to meet the increasing demand for poultry products [3][4]. The consumption of chicken meat and eggs represents cheap, healthy, and quality protein sources in human nutrition globally. However, the breeding programs may subject chickens to physiological dysfunction and immunosuppression by simply focusing on reproduction and or growth rates [5][6], subsequently increasing susceptibility to metabolic disorders and management-associated stressors [7][8]. For example, a laying hen produces approximately 310 eggs annually with a low feed consumption of just 110 g per day [9]. The extreme selection for one trait (production) could affect other biological traits, causing negative impacts on animal health and welfare such as aggression and related injurious behavior [10]. Selection for production increases aggression as, 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 [11][12]. Controversially, selection based on hen behavior may reduce feather pecking, but it may result in an unfavorable correlated selection response, reducing egg production [13].

Currently, the conventional (battery) cage system is the most common housing facility for laying hens in the United Sates (U.S.), which was estimated to be 70.7% of the table egg layer flocks (approximately 231.7 million laying hens) at the end of 2020 [14]. Typically, commercial 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 high stocking density of hens and limited space for hens to display their "natural" behavior (such as foraging, exploration, perching, and nesting) negatively impact their welfare status, resulting in a chronic state of stress [15]. One of the possible strategies 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 system (consisting of a nest, litter bath or scratch area, perches, and abrasive strip) and cage-free systems with or without outdoor access such as aviaries (single- and multiple-tiered) [16]. Although hens housed in the enriched cage system and non-cage systems seem to be possible ways to improve their welfare by displaying some degree of "natural" behavior such as nesting, roosting, and scratching [17][18], there is a high risk of increased exhibition of injurious behavior (feather pecking, aggression, and cannibalism) resulting from large group sizes and social instability [19][20][21][22][23]. Social stress and associated injurious behavior are major concerns in all current housing environments including cage and cage-free systems [19][20].

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

Probiotics are commensal bacteria (“direct-fed microbials”, DFM) that offer potential health beneficial effects to the host’s stress response (acute, chronic or both). Several commercial probiotics have been used in poultry production [24][25], and numerous have shown that probiotics aid chickens in adapting to their environment and improving their health and welfare 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) with antimicrobial function and short chain fatty acids to regulate the activity of intestinal digestive enzymes and energy homeostasis; (3) modulating gut and systemic immunity; (4) restoring the intestinal barrier integrity preventing pathogens from crossing the mucosal epithelium; (5) stimulating the endocrine system and attenuating stress-induced disorders of the HPA and/or sympathetic-adrenal-medullary (SMA) axes via the gut–brain axis; (6) inducing epithelial heat shock proteins to protect cells from oxidative damage; and (7) synthesis and secretion of neurotransmitters such as 5-HT and tryptophan [26][27][28][29][30].

It has been stated in humans and non-human primates that the gut microbiota have potential effects on their hosts’ aggressive behaviors and anxiety symptoms [31][32][33]. In rodent one, germ-free (GF) animals with exaggerated HPA responses to social stress can be normalized by certain probiotics [34][35][36]. In addition, probiotics have successfully attenuated anxiety and depressive behaviors in rat offspring separated from their mother [37][38] and the obsessive-compulsive-like behaviors in mice [39][40]. These results support the psychobiotics theory [41][42] (for example, a special class of probiotics (beneficial bacteria) delivering mental and cognitive health benefits (such as anxiolytic and antidepressant effects) to individuals) and provide a potential to use probiotics as a biotherapeutic strategy for improving a host’s mental and cognitive function in humans and other animals including chickens [43][44][45][46][47][48][49][50]. Probiotics may have similar effects on chicken behavior due to the human–animal transmission occurs during the evolution and ecology of gastrointestinal microbial development (the host–microbial coevolution) [51][52]. Several probiotics have been used in preventing injurious damage in poultry. For example, probiotic *Lactobacillus rhamnosus* JB-1 supplementation (5 × 10^9/mL in drinking water provided from week 19 to week 28) reduces chronic stress (social disruption, physical and manual restraint, and blocking nest boxes and perch usage applied from week 24 to week 26) induced feather pecking and cecal microbiota dysbiosis, along with increased T cell populations in the spleen and cecal tonsils of adult chickens regardless of the genetic lines (HFP and LFP lines) [53]. Probiotic *Lactobacillus rhamnosus* supplement (applied from day 1 to week 9) also counteracted stress-induced decrease in T cells, along with a short-term (from week 10 to week 13) increase in plasma tryptophan and the TRP:(PHE + TYR) ratio (from week 14 to week 15), but without effects on feather pecking in pullets [34]. The TRP:(PHE + TYR) ratio has been used as an indicator of the competition between tryptophan and other amino acids for uptake across the BBB [54]. In addition, the number of feather pecking bouts was positively correlated with intestinal contraction velocity and amplitude in peckers, which can be modulated by administrated *L. rhamnosus* [55]. *Lactobacillus*-based probiotic supplements also reduced stress-associated immobility behavior in rodents during the forced swine test [56]. Parois et al. [57] also reported that probiotic *Pediococcus acidilactici* reduced fearfulness in selected short tonic immobility birds, indicated by a short immobility during the tonic immobility test via regulation of the MGB axis. Reduced fearfulness was also found in a synbiotic one [58]. It consisted of a probiotic (*Enterococcus faecium, Pediococcus acidilactici, Bifidobacterium animalis,* and *Lactobacillus reuteri*) and a prebiotic (fructooligosaccharides). The synbiotic fed broilers had a shorter latency to make the first vocalization with a higher vocalization rate during an isolation test, and a greater number of synbiotic fed birds reached the observer during a touch test. There results revealed a potential strategy to use probiotics to reduce stress response and stress-induced injurious behavior during poultry production. However, large gaps about probiotic functions in improving neuropsychiatric disorders remain, which are affected by multiple factors including the type of probiotic bacteria and duration and dosage of the intervention.

2.1. *Bacillus subtilis*

Bacillus subtilis is one of the three most common species of probiotic products in the U.S. [59] and has been used widely as a functional feed supplement such as in several dairy and non-dairy fermented foods for improving human health and well-being [60][61][62][63]. Similarly, *Bacillus subtilis*-based probiotics have been used as antibiotic growth promoter alternatives in poultry [64][65][66][67]. *Bacillus subtilis* are spore-forming bacteria. They are heat stable, low pH-resistant (the gastric barrier), and tolerate multiple environmental stressors [68][69]. Several mechanisms of actions of *Bacillus sp*p. have been proposed: regulating intestinal microstructure [70] and digestive enzymes [71][72]; synthesizing and releasing antimicrobial and antibiotic compounds [65]; increasing immunity [71][73][74], and neurochemical activities including 5-HT [75][76][77] as well as affecting animal behavior [76]following various stressors. For example, in response stimulations, *Bacillus subtilis* alleviates oxidative stress, provokes a specific biological response, and improves the mood status of hosts via the gut–brain axis [78][79]. In addition, *Bacillus subtilis* can overproduce L-tryptophan [80][81][82], and consequently increase 5-HT in the hypothalamus [83]. Tryptophan functions as an antidepressant and anti-anxiety agent [83][84][85][86] and eliminates nervous tension in mice [87][88]. In one, chickens were used as an animal model to assess whether dietary supplementation of the probiotic *Bacillus subtilis* reduced aggressive behaviors following social challenge [89].

Chickens, as social animals, show fear, depression, and or anxiety in novel environments [52]and show aggression toward others for establishing aa social dominance rank in unfamiliar social groups [90][91][92], which is similar to the rodents used in human psychopharmacological ones [93][94][95]. The paired social ranking-associated behavioral test used in this one [85] has been routinely performed in chicken behavioral analysis [80][96][97]. The rationale 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 [98][99][100][101].

In this [87], the role of the probiotic *Bacillus subtilis* on the aggression in hens of the Dekalb XL (DXL) line was examined. One-day-old female chicks were kept in single-bird cages [89]. The hens at 24-weeks-old were paired based on their body weight for the first behavioral test (pre-probiotic treatment, day 0) in a novel floor pen. To determine the dominant individual per pair, behaviors were video-taped for 2 h immediately after the release of two hens simultaneously into the floor pen. After the test, the subordinate and dominant hens were fed the regular diet or the diet mixed with 250 ppm probiotic (1.0 × 10^6 cfu/g of feed) for two weeks, respectively. The probiotic contained three proprietary strains of *Bacillus subtilis* (Sporulin®, Novus International Inc., Saint Charles, MO, USA). After the treatment (day 14), the second aggression test was conducted within the same pair of hens. The injurious behaviors were detected and analyzed.

It was indicated that compared to their initial levels at day 0, the levels of threat kick were reduced, the frequency of aggressive pecking tended to be lower, and the levels of feather pecking was reduced but without statistical significance in probiotic fed dominant hens. There was no change in injurious behaviors in the regular diet fed subordinate hens between day 0 and day 14. The behavioral changes in probiotic fed dominant hens were correlated with the changes in blood 5-HT concentrations. Post-treatment (day 14), plasma 5-HT levels were reduced toward the levels of the controls (subordinates) in the probiotic fed dominant hens compared to their related levels prior to treatment (day 0). Similarly, the effects of probiotic dietary supplements on behavior have been found in turkeys [98]. The turkey poults fed probiotic *Bacillus amyloliquefaciens* had increased feeding frequency and duration with decreased distress call and aggressive behavior.

The similar relations between reduced aggressive behavioral exhibition and blood 5-HT concentrations were identified in the previous ones [10][102], genetic selection for prevention of social stress-induced feather pecking, and aggression. Compared to MBB mean bad birds (MBB), kind gentle birds (KGB) had lower blood 5-HT concentrations as well as lower concentrations of blood dopamine (DA) and corticosterone (CORT) and a lower heterophil/lymphocyte (H/L) ratio, a stress marker, with lower frequency of injurious pecking [10][103]. Bolhuis et al. [104] also reported that peripheral serotonin activity reflected the predisposition to develop severe feather pecking in laying hens. Similarly, individuals with a lower blood 5-HT level that showed less aggressiveness were found in humans [105][106][107] and canine[108] while an elevated level of blood 5-HT has been revealed in patients with aggressive behavior [109][110] and in aggressive teleost fish [111]. These results provide evidence for serotonergic mediation for aggressive behavior and stress coping strategy; and chicken aggression can be reduced or inhibited by probiotic supplementation by directly or indirectly regulating the serotonergic system.

Whether the changes in blood 5-HT levels in probiotic fed dominant hens represent a similar change in 5-HT concentrations in the brain is unclear as 5-HT cannot pass the blood–brain barrier and is regulated differently between brain neurons and peripheral tissues [112]. The plasma 5-HT is synthesized mainly by the enterochromaffin (EC) cells (also known as Kulchitsky cells), types of enteroendocrine and neuroendocrine cells, of the gut and stored in the platelets [113]. However, it has been proposed that platelet 5-HT uptake is a limited peripheral marker of brain serotonergic synaptosomes [112]. *Lactobacillus plantarum* strain PS128, a dietary probiotic that causes an increase in the levels of striatal 5-HT as well as DA, is correlated with improving anxiety-like behavior in germ-free (GF) mice [113]. Similar results have been obtained from [89][114]. In another one, chickens (broilers) were fed *Bacillus subtilis* from day 1 to day 43. The results indicate that *Bacillus subtilis* fed chickens had higher levels of 5-HT in the raphe nuclei and lower levels of norepinephrine (NE) and DA in the hypothalamus compared to the controls fed a regular diet [113]. Probiotic fed chickens also had improved skeletal traits (bone mineral density, bone mineral content and robusticity index). In one heat stress (32 °C for 10 h), *Bacillus subtilis* fed chickens (broilers) had lower heat stress-related behaviors including panting and wing spreading and inflammatory response in the hypothalamus compared to the controls [82]. Further, however, are needed to examine how the correlations present between injurious behavior and peripheral and or brain 5-HT in probiotic fed chickens.