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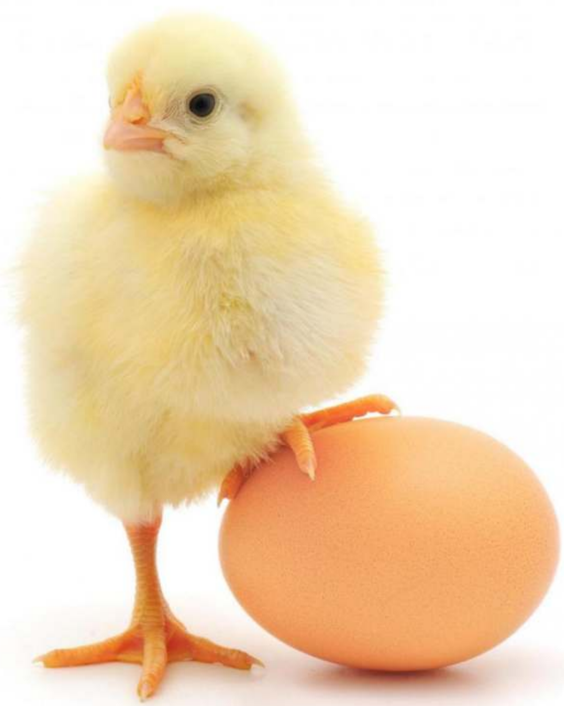

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Research Article
Effect of Direct-fed Microbials, *Bacillus subtilis*, on Production Performance, Serotonin Concentrations and Behavioral Parameters in a Selected Dominant Strain of White Leghorn Hens

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Abstract
Background and Objective: Probiotics modulate stress-induced changes of physiological homeostasis and behavioral exhibition through regulating the microbiota-gut-brain axis. The aim of this study was to assess if dietary supplementation of probiotic, *Bacillus subtilis*, reduces aggressive behaviors in laying hens following social challenge. **Methodology:** Hens (n = 12) of an aggressive stain (Dekalb XL) were housed in single-hen cage prior to the study. At 24 weeks of age, the hens were paired based on their BW to identify the dominance rank within each pair (0 day). The subordinator and dominator of each pair were individually fed a regular layer diet or the diet mixed with 250 ppm probiotic for 2 weeks (days 14). **Results:** Data showed that the exhibition of aggressive behaviors in the regular diet fed subordinates were not affected by the treatment ($p>0.05$), while the frequency of threat kick ($p = 0.04$) was reduced and aggressive pecking ($p = 0.053$) had a tendency to be lower in the probiotic fed dominates compared to the levels at 0 day. Plasma concentrations of serotonin were also reduced in the probiotic fed dominant hens ($p = 0.02$). There were no treatment effects on plasma tryptophan levels, body weight gain and egg production ($p>0.05$, respectively). **Conclusion:** The data indicate that dietary probiotic supplementation could be a useful management tool for preventing aggressive behaviors in laying hens.

Key words: Probiotic, aggression, serotonin, production performance, laying hen

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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INTRODUCTION

Injurious pecking and cannibalism are serious welfare and economic issues for the poultry industry. These harmful behaviors occur in all current housing systems[1], leading to suffering and death in laying hens that have not been beak trimmed. Beak trimming (BT), both hot blade and infrared, has been used as a common practice to prevent or reduce injurious pecking and cannibalism in laying hens. However, there are several welfare concerns regarding BT, such as BT-caused acute pain, chronic pain or both. Current alternatives to BT, such as dimming of lights or providing an enriched environment in a barn are effective in mitigating the performance of these behaviors but do not prevent their performance[2-4]. In addition, injurious behavior-associated social stress may stimulate the activation of the hypothalamic-pituitary-adrenal (HPA) axis, a central stress regulatory system[5] and disturb intestinal bacterial balance[6].

Probiotics, also known as “direct-fed microbial”, have been used as feed additives, to promote animal growth and production performance[7], improve intestinal health[8] and immunity[9,10] in various livestock species and poultry. *Bacillus subtilis (B. subtilis)* is one of the most common probiotics used in poultry as that spores of *B. subtilis* are heat-resistance and tolerate multiple environmental stressors[11]. In addition, *B. subtilis* is a domestically grown bacteria in the caeca of chickens[12]. Several studies have evidenced that dietary supplementation of *B. subtilis* can improve growth performance, feed efficiency, gut health and immunity in broiler chickens[13-15].

Currently, emerging data has shown that changes in the composition of gut microbiota lead to alterations of neuroendocrine function. Probiotics, including *B. subtilis*, reduce inflammation, alleviate the stress response and improve the mood status of hosts[16-18] via the gut-brain axis[17,19,20].

Microorganisms produce neuropeptides and neurotransmitters, including serotonin (5-Hydroxytryptamine, 5-HT)[21,22] through the same or similar biosynthetic pathways of mammals[23]. The serotonergic system is closely involved in modulating physiological homeostasis and behavioral exhibition[24]. Alteration of the serotonergic system leads to an increase in displaying aggressive behaviors and mood disorders in various species of animals[24]. The application of 5-HT successfully relieves the depression syndromes in humans and reduces aggressive behaviors in primates and rodents[24]. Alterations of 5-HT and its correlated metabolites or receptors in the central nervous system (CNS) have also been used as biomarkers for evaluating mood status in humans and aggression in various animals including chickens[25-27].

The majority of 5-HT is synthesized in the enterochromaffin cells (EC) located within the gastrointestinal tract crypts[28,29] and approximately 5% of 5-HT is produced in the neurons within the raphe nuclei of the brainstem. Under normal conditions, the central 5-HT is separated from the peripheral 5-HT, since 5-HT cannot pass the brain-blood-barrier[15,30]. In germ free (GF) mice, gut microbiota modulate the 5-HT synthesis and release at both the brain and peripheral levels directly and or indirectly via the microbiota-host interactions[31-33]. In addition, Sudo *et al.*[34] reported a correlation between the changes of gut microbiota and the function of the HPA axis. Germ-free rats had significantly higher levels of both adrenocorticotropic hormone (ACTH) and corticosterone compared to control rats in response to restraint stress[34]. Emerging evidences have indicated that gut microbiota have important roles in programming of HPA stress reactivity[35] with long-term effects on the physiological and neurobehavioral functions of an individual[36]. In the current study, researchers examined the hypothesis that use of dietary probiotic inclusions to regulate the gut commensal bacteria could be a novel method for controlling injurious behaviors in laying hens via modifying the serotonergic system.

MATERIALS AND METHODS

All hens used in this experiment were housed and cared under the protocol approved by Purdue Animal Care and Use Committee (PACUC). All members involved in hen handling were registered under the PACUC’s protocol (1111000262).

Birds, diets and management: A former commercial line, Dekalb XL (DXL), exhibiting high aggressive behaviors and cannibalism was used in this experiment[27]. The hens were hatched and reared in single-hen cages at the Purdue University Poultry Research Farm located in West Lafayette of Indiana. The hens were fed various regular diets based on their growth phases up to 24 weeks. The nutrients of these diets were or over the request based on the Hy-line W-36 guideline. Feed and water were provided with free access and lighting program was set at 16L:8D with one-foot candle light intensity throughout the experiment.

Table 1: Behaviour 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

Treatment and behavioral observation: At 24 weeks of age, the hens (n = 12) were paired with a similar BW for the first aggression test (pre-treatment, 0 day) in a novel cage allowing 750 cm^2/hen followed the procedure published previously[37,38]. Hen's behaviors were video-taped immediately for 2 h to determine the dominant individual per pair and then the hens were returned to their home cages. Following the behavioral test, subordinate hens were continuously fed the regular layer diet, while dominant hens were fed the regular diet mixed with 250 ppm sporulin (1.0×10^6 CFU g^{-1} of feed) based on the recommendation of the company (Pacific Vet Group-USA, Inc., Fayetteville, AR) for 2 weeks. Sporulin contains three proprietary strains of *B. subtilis.* Post 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 lab procedure published previously (Table 1)[37,38].

Sample collection: Eggs were recorded daily during the study. Hen-day egg production was calculated as: The total number of eggs produced per cage/one hen per cage$\times$100. Body weight was assessed immediately before the first (BW1) and the second (BW2) aggression test, respectively. Body weight gain (BWG) (%) was calculated as the (BW2-BW1)/BW1$\times$100[39].

A 5-mL blood sample per hen was collected into an ethylenediaminetetraacetic acid (EDTA)-coated tube through the brachial vein. The blood samples were centrifuged at 700 rpm for 15 min at 4°C. Plasma were aliquoted into 500 µL tubes and kept at -80°C until further analysis.

Enzyme-linked immunosorbent assay (ELISA): Plasma of 5-HT and tryptophan concentrations were measured in duplicates using commercially available chicken specific ELISA kits (MyBiosource, Inc., San Diego, CA). All samples were analyzed with an absorbance reading of 450 nm by following the manufacturer's protocols.

Statistical analysis: Data of plasma concentrations of 5-HT and tryptophan, BWG and hen-day egg production were subjected to a one-way ANOVA using the MIXED model procedure of the SAS 9.3 software (SAS Institute Inc., Cary, NC). The main effect of treatment was fixed. The individual chicken was the experimental unit. For the behavior data, if data lacked homogenous variances, transformations of BOXCOX were used and the data were reanalyzed. Because statistical trends were similar for both transformed and untransformed data, the untransformed results were presented. Tukey-Kramer was used to partition differences among means due to significant treatment effects. Significant statistical differences were reported when $p\leq 0.05$ and statistical trends were reported when $0.05 < p\leq 0.1$.

RESULTS AND DISCUSSION

Social stress is one of the major concerns of the poultry industry during routine management practices such as mixing unfamiliar chickens during transferring them from grower facilities to layer facilities. Following regrouping, chickens trying to redevelop social rank leads to great display of aggressive pecking and cannibalism[40-42]. The paired social ranking test has been routinely used in chickens[37,38]. The rationale and cellular mechanisms of the test could be similar to the resident-intruder test which is a standardized test used in rodents for detecting social stress-induced aggression and violence[43]. As an evolutionary perspective, aggression in animals is related to survival, growth and reproduction[44-47] and correlates with individual divergence (phenotypic evolution) of physiological function[48-51]. Serotonin, as an ancient chemical, is a key neurotransmitter and modulator in response to various stimulations in almost every living organism[52-54].

The serotonergic system 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) following various stimulations, especially plays an important role in regulating aggressive behaviors[55,56] and mood disorders[57-59]. Abnormalities of blood and brain 5-HT, its metabolite 5-Hydroxyindoleacetic acid (5-HIAA) and precursor tryptophan as well as the density of its various receptors, have been used as indicators or targets in the diagnosis and treatment of psychiatric and compulsive disorders, such as depression and anxiety, in humans and various experimental animal models[24,60,61].

In the CNS, 5-HT functions to inhibit aggression, thereby controlling domestic behaviors[62]. 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[63-65], non-human primates[66,67], rodents[68,69] and birds[27]. In the peripheral system, however, pathophysiological roles of 5-HT in behavioral and motivational regulation are unclear. Decrease, increase and unchanged of blood 5-HT concentrations have all been reported in association with behavioral dysfunctions, including aggressiveness[70-72]. 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. The present data showed that prior to the treatment (0 day), plasma 5-HT levels were higher (26% increase) in the dominant hens than that of subordinate hens but did not reach statistical significance (p = 0.24). $Dominant_{5\text{-}HT}$ = 17.46 ng mL^{-1}, $subordinate_{5\text{-}HT}$ = 13.87 ng mL^{-1}). This finding is in agreement with the results reported previously[73,74]. In those studies, higher plasma levels of 5-HT were detected in hens from a high aggressive strain (mean bad bird, MBB, selected for both low productivity and low longevity resulting from injurious pecking and cannibalism) compared to hens from a low aggressive strain (kinder gentler bird, KGB, selected for both high productivity and high longevity). In addition, Bolhuis *et al.*[75] proposed that blood 5-HT activity 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, i.e., a lower blood 5-HT level was associated with less aggressive individuals in humans[71,76] and canine[72], while an elevated blood 5-HT level was determined in aggressive patients[60,63] and teleost fish[77].

Post-treatment (day 14), plasma 5-HT levels were reduced in the probiotic fed dominant hens (p = 0.02) but not in subordinate hens fed a regular diet (p = 0.88) compared to the related levels prior to treatment (0 day, Fig. 1). Although, the resources of enhanced 5-HT in the probiotic fed dominant hens were not determined in the current study, some strains of probiotics have the potential to produce a large array of neuroactive factors (neuropeptides and neurotransmitters) including 5-HT[78,79]. In addition, Wikoff *et al.*[31] reported that conventional mice had lower concentrations of 5-HT compared to GF mice. GF mice also had an exaggerated stress response[34] with anxiety-like behaviors[80]. These abnormal behaviors in GF mice can be inhibited or reduced by feeding probiotics[81] or transplanting fecal samples of conventional mice[82]. The current and previous results indicate that normal health gut microbiota play an important role in regulating social stress and stress-associated behaviors.

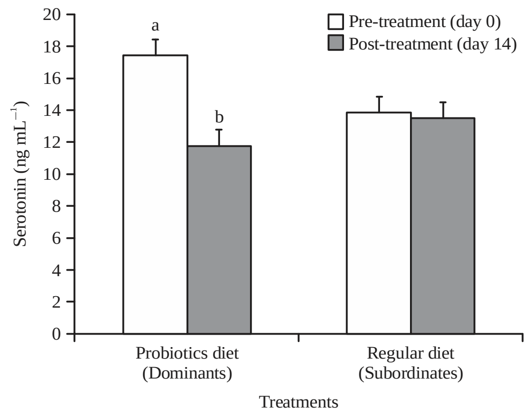

Fig. 1: Plasma serotonin (5-HT) levels at day 0 (pre-treatment) and day 14 (post-treatment) in probiotic fed dominant hens and regular diet fed subordinate hens. Compared to subordinate hens, plasma 5-HT concentrations were higher in dominant hens at day 0 but without statistical different (p = 0.24); 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)

Whether the changes of blood 5-HT levels in probiotic fed hens before (day 0) and after the treatment (day 14) represent a similar changes of 5-HT concentrations occurred in the brain is unclear, as 5-HT cannot pass the brain-blood-barrier[30] and is regulated differently between brain neurons and peripheral tissues[30,83,84]. The plasma 5-HT is synthesized mainly by the EC cells of the gut and stored in the platelets[85]. However, it has been proposed that platelet 5-HT uptake is a peripheral marker of brain 5-HT[72]. Further studies are needed to examine the regulations of peripheral 5-HT and CNS 5-HT in probiotic treated hens.

The gut commensal microflora may have an indirect effect on 5-HT synthesis by regulating tryptophan metabolism. Tryptophan, as an essential amino acid, is a precursor of 5-HT. Tryptophan degradation is mainly through the kynurenine pathway. The pathway regulates over 95% of tryptophan in the peripheral system and is functionally mediated by gut mycobacteria and probiotics[17,86,87]. In the present study, the tryptophan level was not significantly affected in probiotic fed hens (p = 0.35) but the initial level of tryptophan in dominant

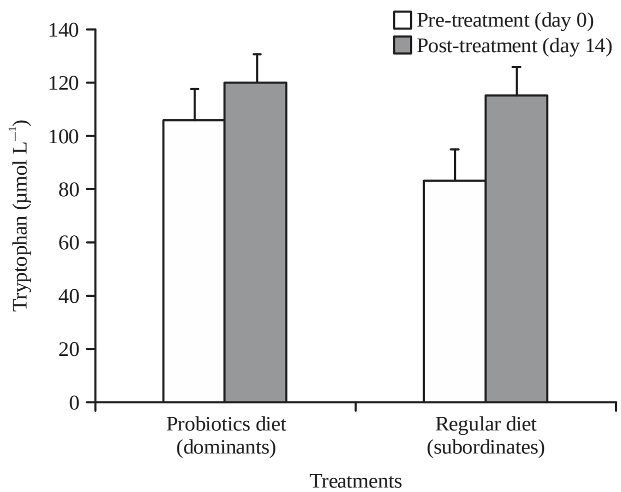

Fig. 2: Plasma tryptophan levels at day 0 (before-treatment) and day 14 (post-treatment) in probiotic fed hens and regular diet fed hens. Prior to treatment, dominant hens had higher tryptophan concentrations compared to subordinates but the difference did not reach statistical difference (p = 0.21). There were no treatment effects on tryptophan concentrations in both probiotic fed hens and regular diet fed hens (p>0.05, respectively)

hens was approximately 28% higher than the subdominant hens (p = 0.21) (Fig. 2). 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 indirectly regulate 5-HT synthesis in the peripheral system.

Behavioral changes in dominant hens were correlated with the changes of blood 5-HT following treatment. Originally dominant hens became calmed after fed with probiotic, resulting in that all measured behavioral patterns were reversed between dominants and subordinates (Fig. 3). In the probiotic fed dominant hens, the frequency of threat kick were reduced (Fig. 3a, p = 0.04), the frequency of aggressive pecking tended to decrease (Fig. 3b, p = 0.053) and the frequency of feather pecking was reduced but without statistical significance (Fig. 3c. 58%, p > 0.05) compared to the levels of injurious behaviors observed on day 0. There were no changes in the performance of injurious behaviors in the regular diet fed subordinate hens between day 0 and day 14 (Fig. 3a-d). The cellular mechanisms underlying these behavior changes in probiotic fed dominant hens are unclear but could be similar to the ones proposed in humans and rodents, the probiotic-manipulated commensal bacteria may directly release neuroendocrine factors[78] which indirectly affect the neurotransmitters and neuronal regulators, such as tryptophan, through the microbiota-gut-brain axis[17,35,80,88]. The effects of probiotics, including *B. subtilis*, on behavioral patterns have been proved by a growing investigations conducted on GF mice. Bercik *et al.*[82] 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). In addition, probiotics have therapeutic effects on neurodevelopmental disorders, for example, reduced anxiety-like behaviors by providing *Lactobacillus helveticus*, *Mycobacterium vaccae* and/or *Bifidobacterium* strains[82,89,90] and alleviated autism related stereotypic behaviors by treating with *Bacteroides fragilis*[91].

In the current study, BWG 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 supplemental probiotics on daily weight gain, finish BW and feed conversion rate in broilers[92-94], turkeys[95] and pigs[96,97]. Several studies also reported the improvement of egg production in hens fed probiotic diets[98-100]. The underlying mechanisms may be related to the beneficial bacterial growth in the gastrointestinal tract facilitates the fermentation process by which improves the digestion and utilization of nutrients in animals[92,101]. However, the beneficial effects on growth performance may be affected by the bacteria strains, preparation process, dosage, chicken's age and genetic type[102,103]. 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.

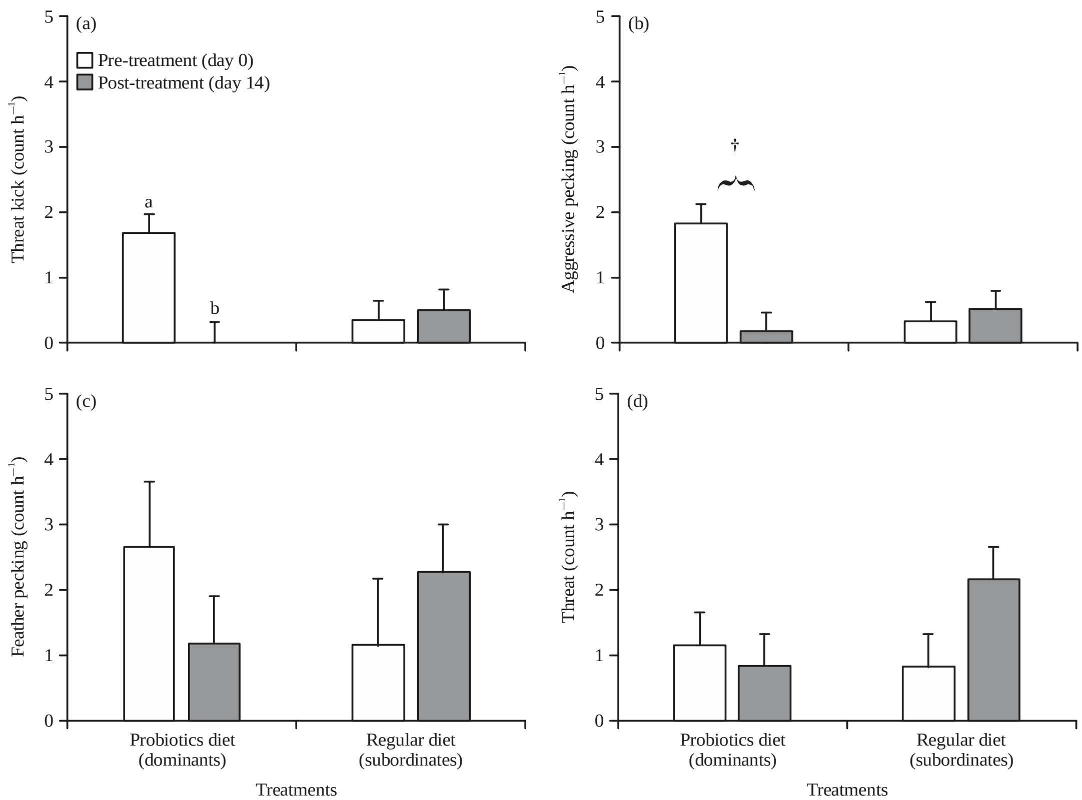

Fig. 3(a-d): Frequency of aggressive behaviors at day 0 (before-treatment) and day 14 (post-treatment) in probiotic fed hens and regular diet fed hens followed the paired social test. The exhibitions of aggressive behaviors in the regular diet fed subordinates were not affected by treatment (p>0.05, 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. The treatment effects resulted in that the display of 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 (p<0.05, p≤0.10)

CONCLUSION

Present study 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.

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