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The Influence of Dietary Synbiotic on Agonistic Behavior, Stress, and Brain Monoamines via Modulation of the Microbiota–Gut–Brain Axis in Laying Hens

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Abstract: A complex system of neural pathways, collectively known as the microbiota–gut–brain (MGB) axis, interconnects the gut microbiota, the gastrointestinal system, and the brain along with its periphery. Previous studies have demonstrated that modulation of the MGB axis can influence stress-related behaviors such as anxiety. This connection becomes apparent in scenarios like agonistic behavior in laying hens, which is characterized by aggressive head and feather pecks, that can ultimately result in cannibalism and death. The objective was to examine the effects of a dietary synbiotic on agonistic behavior, plasma and brain monoamines, stress parameters, and cecal microbiota counts via modulation of the MGB axis. A total of 396 W36 Hy-Line laying hens were provided at random with a control (CON: basal diet) or treatment (SYN: basal diet supplemented with synbiotic) diet from 50 to 60 weeks old (nine pens/treatment, 22 birds/pen). Blood samples and video recordings (three consecutive days/week) were taken at 50 and 60 weeks. At 60 weeks, three hens/pen were euthanized for brain and cecal microbiota collection. Threatening, fighting, head, body, and feather pecking all occurred less frequently at 60 weeks in the SYN group ($p < 0.05$). Plasma corticosterone, adrenocorticotropic hormone, dopamine, and serotonin were significantly lower while tryptophan and 5-hydroxyindoleacetic acid were significantly higher in birds from the SYN group ($p < 0.05$). Significant differences in serotonin, 5-hydroxyindoleacetic acid, dopamine, homovanillic acid, and 3,4-dihydroxyphenylacetic acid were observed in the hypothalamus, hippocampus, and amygdala of the brain. Serotonin and dopamine turnover rates were significantly different in all three regions of the brain ($p < 0.05$). Cecal counts of *Lactobacillus* and *Bifidobacterium* were significantly higher in the SYN group ($p < 0.05$). Synbiotic supplementation resulted in many significant differences, indicating activation of the serotonergic systems and modulation of both the MGB axis and HPA axis with positive effects on welfare and stress.

Keywords: synbiotics; laying hen; agonistic behavior; microbiota–gut–brain axis; welfare; serotonergic system; dopaminergic system

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1. Introduction

There exists a complex system of neural pathways that interconnect the gut microbiota and gastrointestinal system with the brain and its periphery, collectively called the microbiota–gut–brain (MGB) axis [1,2]. The overview of the pathway begins with external factors like disease, nutrition, environment, and genetics acting on the gut microbiota. These external factors may influence the composition of the microbiota, which then can

directly influence cell structure or indirectly synthesize neurotransmitters and induce the synthesis of neurotransmitters [1,3]. They can be carried via the bloodstream to the brain where effects such as neurogenesis or activation of the hypothalamic–pituitary–adrenal (HPA) axis can occur [3]. This system includes many specific nervous system groups like the autonomic nervous system (ANS) and the hypothalamic–pituitary–adrenal (HPA) axis [2]. Stress hormones like cortisol can be synthesized and released by activation of the HPA axis. Synthesis of short-chain fatty acids and modulation of the neuroendocrine system are among several ways that the nervous system can communicate [1]. These complex interactions with the brain can ultimately influence factors like memory, stress behavior, and feeding behavior [3].

Previous studies have shown that stress-related behaviors like anxiety and cognition can be affected by MGB axis modulation [1]. In humans, the MGB axis contains mechanisms that impact disorders like Alzheimer's disease and major depressive disorder but are not fully understood [4]. In animal models, conditions like obesity and anxiety behavior are affected by the MGB axis [3].

Prebiotics and probiotics are two classes of alternative feed additives that provide nutrients to the gut microbiota and specific microorganisms to the gut microbiota, respectively [5]. Synbiotics, carefully curated combinations of prebiotics and probiotics, are chosen for their capacity to collaborate synergistically, thereby potentially augmenting their efficacy for the host organism [6]. Prebiotics are typically oligosaccharides, while probiotics, also known as direct-fed microbials, are commonly sourced from the genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* [7]. One study found supplementing *Bacillus amyloliquefaciens* to turkey poults increased feeding behavior and duration while decreasing agonistic behavior like fighting and biting [8]. Another study found changes in serotonin, norepinephrine, and dopamine levels in different parts of the broiler brain when supplemented with *Bacillus subtilis* [9]. As recently as 2023, a previous study found differences in the cecal microbiota profile, serotonin metabolism, and immune response, which influence low and high feather-pecking genetic lines [10]. However, no studies to our knowledge have looked at the effect of a synbiotic on agonistic behavior, brain monoamine levels, and cecal microbial profiles simultaneously in laying hens.

The objective of this study was to examine the effects of a dietary synbiotic on agonistic behavior via modulation of the MGB axis in laying hens. We hypothesize that the group administered the synbiotic will demonstrate a decrease in the frequency of agonistic behavior, lower levels of brain monoamines, and the highest counts of cecal-beneficial microbiota.

2. Materials and Methods

2.1. Ethics

This project was given approval under protocol #AUP2021-0068 by Clemson University's IACUC (Institutional Animal Care and Use Committee).

2.2. Environment

At the Morgan Poultry Center (Clemson, SC, USA), a poultry house with ventilation and temperature control was utilized for this experiment. A total of 396 W36 Hy-Line laying hens (50 to 60 weeks of age) were randomly allocated across 18 pens (22 birds/pen) from 50 to 60 weeks of age. Each pen was 5.04 m^2, with approximately 7.6 cm of clean pine wood shavings covering the floor. Feed was given in moveable circular hanging feeders ad libitum, and water was available in automatic cup drinkers ad libitum. Nest boxes were provided at the back of each pen, and the ambient temperature was set to 23.8 °C, with an additional ceiling fan to provide air circulation. Following the standard breed guidelines, the lighting schedule was set to 16 h of light to 8 h of no light. A commercial layer feed product was used as the basal diet composed of a mainly ground corn crumble with guaranteed analysis in Table 1 (16% Layer Crumbles, Tucker Milling, LLC, Guntersville, AL, USA).

Table 1. Guaranteed analysis of layer crumble.

Nutrient	Percentage
Crude protein (min.)	16
Lysine (min.)	0.85
Methionine (min.)	0.36
Crude fat (min.)	3
Crude fiber (max.)	6.5
Phosphorus (min.)	0.6
Calcium (min.–max.)	4.1–4.4
Salt (min.–max.)	0.45–0.8

2.3. Treatments

A commercial product was mixed thoroughly with the basal diet as the synbiotic supplementation called PoultryStar® me^{us} (product code 5016924, DSM Nutritional Products Ltd., Kaiseraugst, Switzerland). The strains within the synbiotic were *Enterococcus faecium*, *Pediococcus acidilacticii*, *Bifidobacterium animalis*, and *Lactobacillus reuteri*, with a minimum guaranteed analysis is 2.0×10^{11} CFU/kg. This resulted in 2 treatment groups (8 pens/treatment): the control (CON; receives basal diet only) and a treatment group (TRT; receives basal diet with the synbiotic supplementation 1 kg/metric ton, as per the manufacturer's recommendation)

2.4. Behavior

Each pen was equipped with a single camera on a closed-circuit system. The computer was programmed for the corresponding date and time. Recordings were set for continuous 24 h intervals for 3 days at 50 and 60 weeks of age. Data were stored within the hard drive of the system and transferred to external hard drives for storage.

Instantaneous scan sampling occurred at 50 and 60 weeks old using BORIS event-logging software v. 8.25 [11]. Scans occurred for a period of 5 s every five minutes in a 2 h time period. Observations were conducted for 3 time periods/day (morning, midday, and evening) over 3 consecutive days (n = 648 observations/timepoint/treatment). The morning observation started immediately after the lights went on, excluding the 30 min sunrise (gradual increase in the lighting to mimic sunrise). The evening observation was conducted two hours before the light went off, excluding the 30 min sunset (dimming light to mimic sunset before complete darkness). Midday observation was around the midpoint of the light period, approximately 14:00 to 16:00. The frequency of agonistic behaviors was recorded, and detailed descriptions of each behavior are listed in the ethogram in Table 2. Any observation points that contained a person or people in the pen were excluded from the totals.

Table 2. Behavior ethogram of agonistic behaviors. Adapted from [12–15].

Agonistic Behavior	Description
Head peck	A firm peck to the head with the receiver flinching
Fighting	Two hens actively jumping and pecking at one other.
Body peck	One hen using her beak to aggressively peck the body of a hen.
Threatening	Two hens in an upright, erect position with pecks delivered; the recipient often has an avoidance response
Feather peck	One hen aggressively pecking one bird and grabbing and/or pulling out feathers.

2.5. Blood

At 50 and 60 weeks of age, laying hens (n = 54) were randomly selected. Samples at 50 weeks were taken approximately 12 h after the synbiotic treatment began to establish the baseline levels of all measurements. The brachial wing vein was used for blood sampling and collected immediately into tubes treated with EDTA and transported on ice to the

lab. Samples were spun in a centrifuge (for 10 min at 5590 rcf and 4 °C) and the top layer of plasma was separated. Plasma was analyzed for corticosterone (CORT), adrenocorticotropic hormone (ACTH), tryptophan (TRP), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) using commercial ELISA kits (My BioSource, San Diego, CA, USA; Shanghai Jianglai Biotechnology CO., Ltd., Shanghai, China; YLA0020CH, YLA0011QU, YLA1546RA, YLA0248RA, YLA0337RA, YLC0076GE, YLC0378MO) and following the respective kit instructions.

2.6. Brain and Cecal Collection

Adapted from [16], laying hens (n = 54) were euthanized via CO_2 chamber to reduce the risk of brain-stem damage. The birds were transported immediately on ice to the lab. The birds were first weighed on a calibrated scale. Heads were first removed intact with approximately 5 cm of neck attached. The remaining body was used for dissection of the cecal collection.

2.6.1. Brain Sample Preparation

Feathers and skin around the neck and head were first removed. Using a sharp Metzenbaum Scissor, the beak was cut along the frontal plane, and the periorbital tissue was dissected to successfully extirpate the eyeballs and access the top of the skull. The brain cavity was opened from the optic nerve connection and the top of the skull was removed using Iris Scissors. The neck was separated at the C1–C2 connection, so the brainstem was visible at the foramen magnum. Using a small, sharp tip, curved Mayo Scissor, with the tip pointed in the superior direction into the foramen magnum to remove the fascia surrounding the brain, the skull was slowly cut into and removed in pieces. Whole brain samples were separated, removed, placed in 10 mL plastic tubes with n-heptane, and deep frozen at −80 °C until analyzed. Following [17], brain slices at a thickness of 400 µm were prepared using a cryostat at −10 °C. The amygdala, hippocampus, and hypothalamus were identified using diagrams from [16,18].

2.6.2. Determination of Brain Monoamines

Brain monoamine levels were determined using the HPLC (high-performance liquid chromatography) method described by [18]. An amount of five µM of clorgyline, 5 µg/mL glutathione, and 1.2 µM of N-methylserotonin were added to the sample and homogenized in ice-cold conditions using a sonicator. Then, 20 µL 2 M $HClO_4$ was stirred into the sample to create 80 µL homogenate and placed in ice water for 15 min. The solution was centrifuged at 15,000× *g* for 15 min at 4 °C and diluted 10 times with water. Along with electrochemical detection, HPLC was used to determine the levels of serotonin (5-HT), dopamine (DA), the metabolite of serotonin called 5-hydroxyindoleacetic acid (5-HIAA), and metabolites of dopamine called homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC). Turnover of serotonin was determined as (5-HIAA/5-HT) and turnover of dopamine was measured as (HVA + DOPAC)/DA). The HPLC was conducted with an AS300 autosampler with a P100 pump, and the Atlas 2003 data-acquisition program was used (Thermo Separation Products, Waltham, MA, USA). A solution of 45 µL/L dibutylamine, 10% methanol, 50 mM citric acid, 0.1 mM EDTA, 50 mM phosphoric acid, and 77 mg/L 1-octanesulfonic acid sodium salt were added to the mobile phase. Sodium hydroxide was used to buffer the pH to 3.4. The flow rate was 0.8 mL/min, and separation occurred at 45 °C. Concentrations were calculated by a comparison of standards, both internal and external, with the protein of each sample determined by the DC protein Assay (Bio-Rad, Hercules, CA, USA). Concentrations of monoamines were expressed as nmol/g of protein.

2.6.3. Cecal Contents

Using poultry shears, the abdominal cavity was exposed. Once the digestive tract was located, the lower half, which included the ileum, ileocecal junction, and ceca, was gently removed from the peritoneal membranes. Then, a second person assisted the primary

person in equipping sterile gloves. Using autoclaved scissors and a spatula, one cecum was opened, and the contents were placed into a sterile Eppendorf tube. A solution of sterile 0.9% sodium chloride solution was added at an approximately 1:1 ratio of sample. New gloves, scissors, pipette, and spatula were used for every sample.

Following the methods by [19], the contents were emptied into a sterile bag and further diluted 10 fold with sterile 0.9% sodium chloride. The mixture was homogenized for 3 min via a bag mixer. A serial dilution occurred from 10^{-1} to 10^{-7}. One-tenth of each sample was coated in the appropriate agar media. Rogosa agar was used for *Lactobacillus* spp., Beerens agar was used for *Bifidobacterium* spp., reinforced clostridial agar was used for *Clostridium* spp., and MacConkey agar was used for *Coliforms* spp. Samples for *Coliforms* spp. were incubated aerobically for 24 h at 37 °C, while the other three media were incubated in sealed anaerobic jars for 48 h at 37 °C. The number of colonies was expressed as log 10 CFU per gram fresh sample.

3. Statistical Analysis

Statistics were conducted using the R software s'tats' package (version 4.3.2, R Core Team, 2023). The "psych" package was used for descriptive statistics. To assess the normality of data, the Shapiro–Wilk test ($p > 0.05$), using the "shapiro.test" package, and a visual inspection of the histograms, using the "hist." package, were conducted, and the data were concluded to have a normal distribution. With the family set to "Poisson", a generalized linear mixed model using the "lme4" package was used to describe the influence of symbiotic supplementation on behaviors; blood, brain, and cecal metabolites; and across ages (in weeks) and all interactions. The main effects were dietary treatment and week of age, with unit and individual birds where possible, as random effects; $p \leq 0.05$ was set as the level of significance using the following model:

$$Y_{ijkl} = \mu + B_i + T_j + BT_{ij} + C_{kl} + e_{ijkl}$$

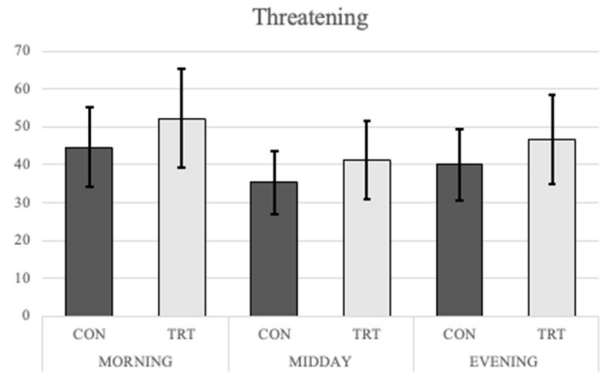
where Y_{ijkl} is the dependent variable, μ is the overall mean, B_i is the effect of the diet, T_j is the effect of age in weeks, BT_{ij} is the interaction between B_i and T_j (diet and age, respectively), C_{kl} is the effect of individual bird within B_i, and across T_j, and e_{ijkl} is the residual error.

Tukey's honestly significant difference (HSD) multiple comparison procedure using the "multcomp" package was used to further analyze statistically significant results. Statistically significant results from Tukey's HSD tests are indicated in figures or tables by different superscript letters. The results are shown as mean ± standard error of the mean (SEM) with p values of pairwise comparisons.

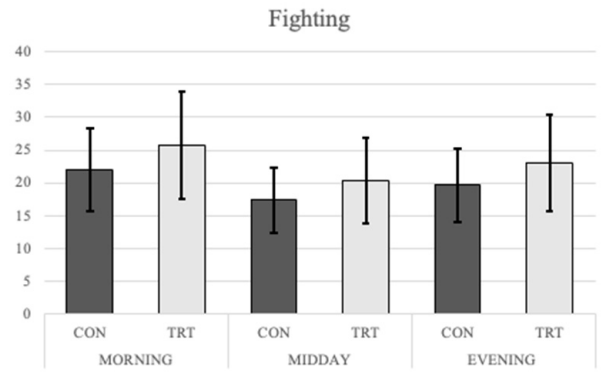
4. Results

4.1. Behavior

Our findings of agonistic behavior are presented in Figures 1a–e and 2a–e. No significant difference between treatments was observed at 50 weeks ($p > 0.05$). At 60 weeks, all behaviors occurred significantly more often in the CON group compared to the SYN. Threatening occurred more frequently in the CON group in relation to the SYN group in the morning ($p = 0.036$), midday ($p = 0.022$), and evening ($p = 0.043$) periods. The SYN group had less frequency of fighting compared to the CON group during the morning ($p = 0.031$), midday ($p = 0.035$), and evening ($p = 0.038$). Pecking occurred more frequently in the CON group in comparison to the SYN group in the morning ($p = 0.029$), midday ($p = 0.043$), and evening ($p = 0.039$) periods. Birds in the SYN group head-pecked less frequently than birds in the CON group during the morning ($p = 0.033$), midday ($p = 0.036$), and evening ($p = 0.036$) times. Feather pecking occurred less frequently in the SYN group versus the CON group in the morning ($p = 0.041$), midday ($p = 0.039$), and evening ($p = 0.029$) times.



(**a**)



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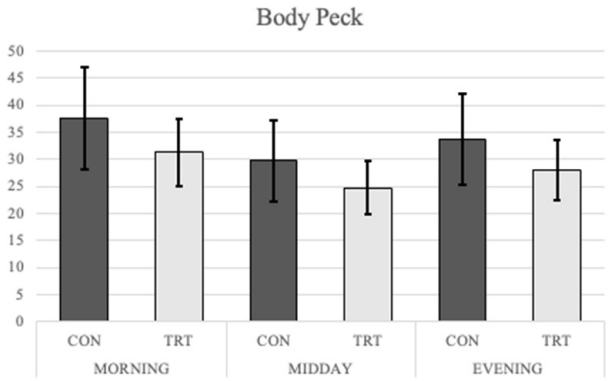

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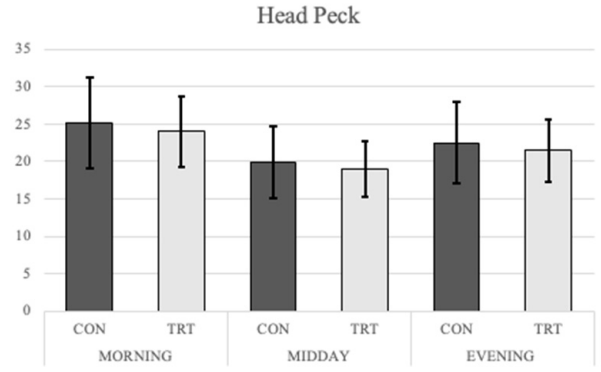

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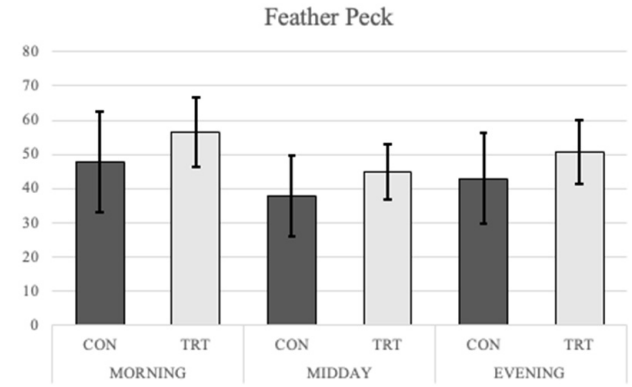


(**e**)

Figure 1. Frequencies of agonistic behaviors at 50 weeks of age (n = 648/timepoint/treatment). Data presented as average frequency of the behavior observed in the 2 h period. (**a**) Frequency of threatening at 3 timepoints; (**b**) frequency of fighting at 3 timepoints; (**c**) frequency of body pecks at 3 timepoints; (**d**) average occurrences of head pecks at 3 points during the light period; and (**e**) average occurrences of feather pecks at 3 points during the light.

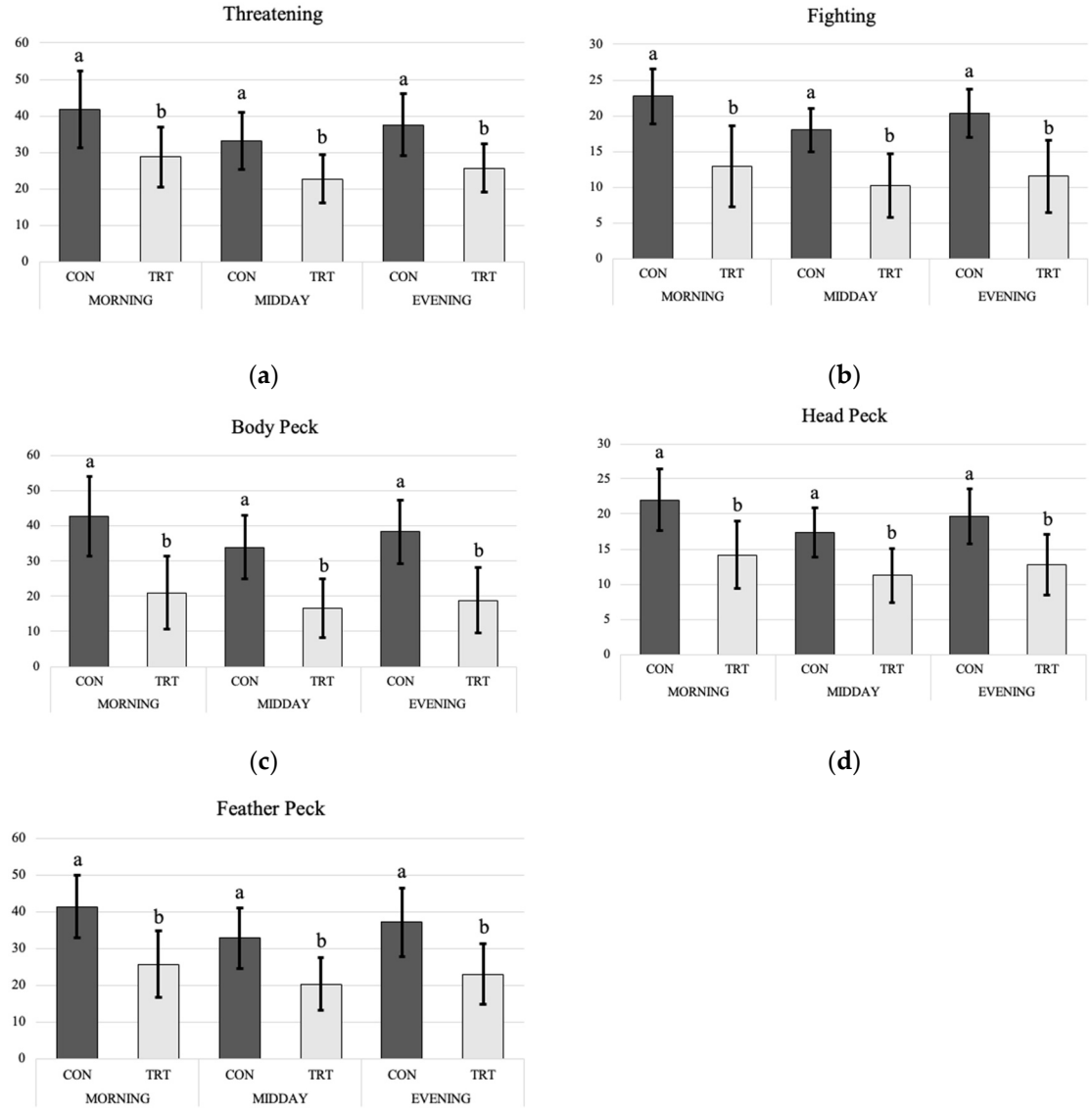


Figure 2. Frequencies of agonistic behaviors at 60 weeks of age (n = 648/timepoint/treatment). Data presented as average frequency of the behavior observed in the 2 h period. (**a**) Average occurrences of threatening at 3 timepoints; (**b**) average occurrences of fighting at 3 timepoints; (**c**) frequency of body pecks at 3 timepoints; (**d**) frequency of head pecks at 3 points during the light period; and (**e**) frequency of feather pecks at 3 points during the light. [a,b] Means with different superscripts differ at $p < 0.05$.

4.2. Blood

The results of plasma samples are presented in Table 3. No significant differences were seen at 50 weeks of age ($p > 0.05$). All values at 60 weeks of age were significantly different ($p < 0.05$), while there were no statistically significant results due to the treatment effect at week 50. The levels of corticosterone were significantly lower in the SYN group compared to the CON group at 60 weeks of age ($p = 0.026$). The concentration of ACTH was significantly lower in the SYN group relative to the CON group at 60 weeks ($p = 0.006$). The levels of tryptophan were significantly higher in SYN birds versus CON birds at 60 weeks ($p = 0.001$). The concentrations of DA were significantly higher in the CON group compared to the SYN group at 60 weeks ($p = 0.016$). The CON group had a significantly higher level of 5-HT versus the SYN group at 60 weeks ($p = 0.003$; Table 3). The levels of 5-HIAA were

significantly lower in the SYN group in relation to the CON group at 60 weeks ($p = 0.012$; Table 3). The turnover rate of 5-HT was significantly higher in the SYN group compared to the CON group at 60 weeks ($p = 0.019$).

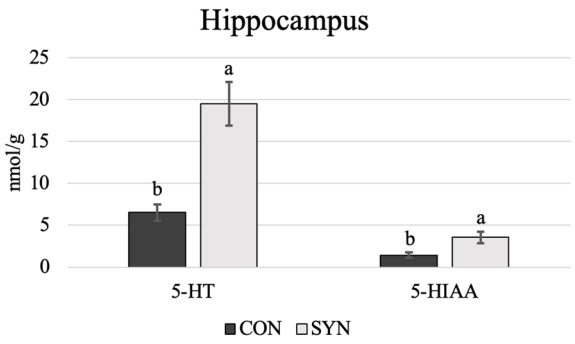
Table 3. Blood plasma sample results at 50 and 60 weeks of age.

	50 Weeks				
	CON	**SYN**	**CON SEM**	**SYN SEM**	***p*-Value**
CORT [1]	36.52	39.26	4.52	3.69	0.230
ACTH [2]	13.88	15.58	1.36	2.06	0.152
TRP [1]	38.69	41.52	6.23	7.03	0.356
DA [1]	131.25	143.89	16.52	11.55	0.859
5-HT [1]	142.36	139.55	9.69	12.85	0.659
5-HIAA [1]	13.52	12.56	2.63	6.25	0.538
5-HT turnover	0.11	0.13	0.06	0.09	0.693
	60 Weeks				
	CON	**SYN**	**CON SEM**	**SYN SEM**	***p*-Value**
CORT [1]	42.36 [a]	21.04 [b]	1.56	1.11	0.026
ACTH [2]	15.96 [a]	7.18 [b]	22.47	11.98	0.006
TRP [1]	43.33 [b]	70.96 [a]	8.47	4.08	0.001
DA [1]	178.50 [a]	114.41 [b]	19.00	11.26	0.016
5-HT [1]	172.26 [a]	118.19 [b]	10.95	11.25	0.003
5-HIAA [1]	15.28 [b]	26.17 [a]	3.18	5.41	0.012
5-HT turnover	0.13 [b]	0.26 [a]	0.09	0.08	0.001

[1] Units presented as ng/mL. [2] Units presented as pg/mL. [a,b] Means with different superscripts differ at $p < 0.05$.

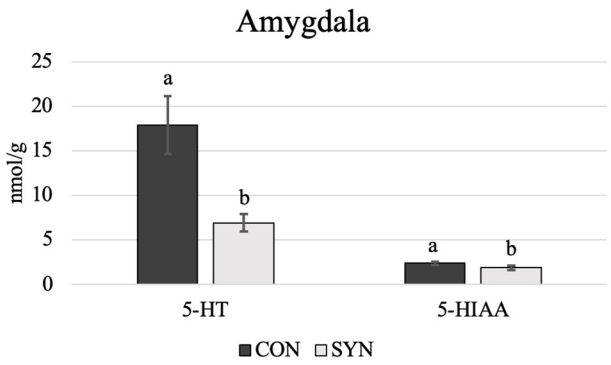
4.3. Brain Monoamines

The results for serotonin and 5-HIAA levels are presented in Figure 3a–c. The concentrations of serotonin in the hippocampus were significantly higher in the SYN group compared to the CON group ($p = 0.013$, Figure 3a). The concentrations of serotonin were significantly lower in the SYN group compared to the CON group in the amygdala ($p = 0.039$, Figure 3b) and the hypothalamus ($p = 0.026$, Figure 3c). The levels of 5-HIAA were significantly higher in the SYN group compared to the control group in the hippocampus ($p = 0.021$). Concentrations of 5-HIAA were significantly lower in the SYN group compared to the CON group in the amygdala ($p = 0.032$) and hypothalamus ($p = 0.001$).



(**a**)

Figure 3. *Cont.*



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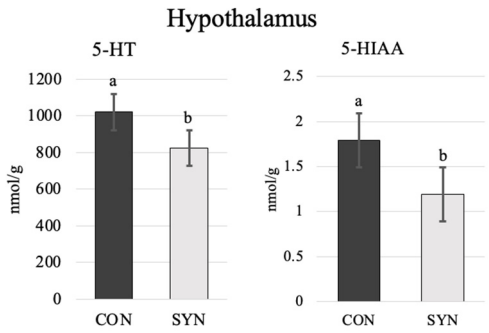
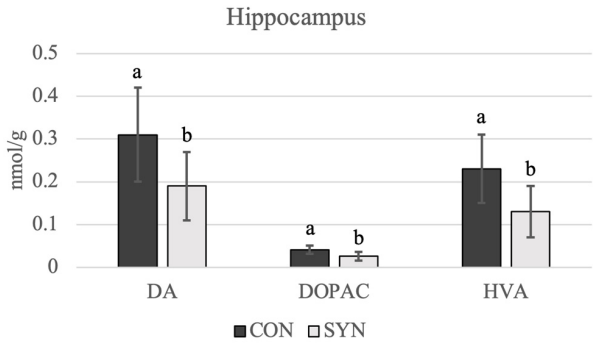


(**c**)

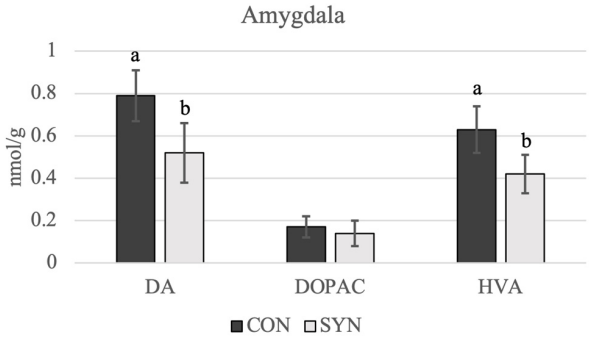
Figure 3. Serotonin and 5-HIAA levels in three parts of the brain. Concentrations are in units of nmol per gram of brain tissue. (**a**) Levels of 5-HT and 5-HIAA in the hippocampus; (**b**) levels of 5-HT and 5-HIAA in the amygdala; and (**c**) levels of 5-HT and 5-HIAA in the hypothalamus. [a,b] Means with different superscripts differ at $p < 0.05$.

The levels of dopamine, DOPAC, and HVA are presented in Figure 4a–c. Concentrations of dopamine were significantly lower in the SYN group in comparison to the CON group in the hippocampus ($p = 0.032$, Figure 4a) and amygdala ($p = 0.021$, Figure 4b). No differences were found in the DA levels in the hypothalamus across treatments ($p = 0.052$, Figure 4c). The concentration of DOPAC was significantly lower in the SYN group versus the CON group in the hippocampus ($p = 0.032$). No differences were observed in the DOPAC levels in the amygdala ($p = 0.053$) or the hypothalamus ($p = 0.052$) across treatments. The concentrations of HVA were significantly lower in the SYN group compared to the CON group in the hippocampus ($p = 0.022$), amygdala ($p = 0.042$), and hypothalamus ($p = 0.041$).



(**a**)

Figure 4. *Cont.*



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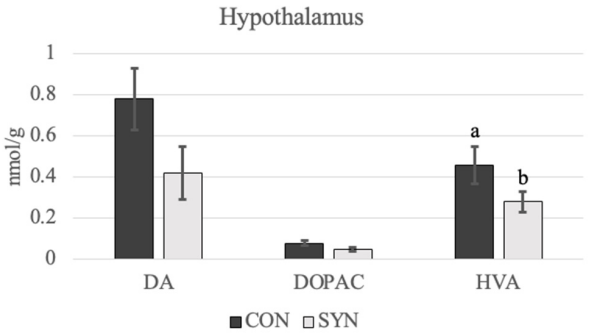

(**c**)

Figure 4. Dopamine, DOPAC, and HVA levels in three parts of the brain. Concentrations are in units of nmol per gram of brain tissue. (**a**) Levels of DA, DOPAC, and HVA in the hippocampus; (**b**) levels of DA, DOPAC, and HVA HIAA in the amygdala; and (**c**) levels of DA, DOPAC, and HVA in the hypothalamus. [a,b] Means with different superscripts differ at $p < 0.05$.

Rates of turnover for serotonin and dopamine are presented in Table 4. The turnover rate of 5-HT was significantly higher in the CON group compared to the SYN group in the hippocampus ($p = 0.001$) and hypothalamus ($p = 0.032$). The turnover rate of 5-HT was significantly higher in the SYN group compared to the CON group in the amygdala ($p = 0.026$). The turnover of dopamine was significantly higher in the CON group compared to the SYN group in the hippocampus ($p = 0.033$) and amygdala ($p = 0.036$; Table 4). The dopamine turnover rate was significantly lower in the CON group compared to the SYN group in the hypothalamus ($p = 0.029$).

Table 4. Turnover rate of serotonin and dopamine in 3 parts of the brain.

5-HT	CON	SYN	CON SEM	SYN SEM	*p*-Value
Hippocampus	0.24 [a]	0.17 [b]	0.09	0.08	0.001
Amygdala	0.12 [b]	0.27 [a]	0.03	0.08	0.026
Hypothalamus	0.011 [a]	0.001 [b]	0.001	0.001	0.032
DA	**CON**	**SYN**	**CON SEM**	**SYN SEM**	***p*-Value**
Hippocampus	0.99 [a]	0.81 [b]	0.08	0.05	0.033
Amygdala	1.19 [a]	1.01 [b]	0.07	0.06	0.036
Hypothalamus	0.61 [b]	0.79 [a]	0.02	0.04	0.029

[a,b] Means with different superscripts differ at $p < 0.05$.

4.4. Cecal Microbiota Contents

The results of the cecal microbiota count are presented in Figure 5. The counts of *Lactobacillus* species were significantly higher in the SYN group compared to the CON group ($p = 0.036$). The counts of the *Bifidobacterium* were significantly higher in the synbiotic

group compared to the control group ($p = 0.039$). No statistical differences were observed for the *Clostridium* species ($p = 0.262$) or Coliforms species ($p = 0.109$) counts.

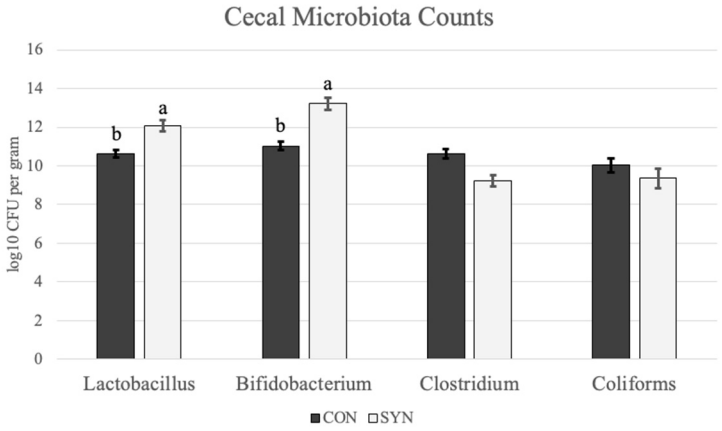

Figure 5. Microbiota counts of selected species from the ceca of laying hens at 60 weeks old. [a,b] Means with different superscripts differ at $p < 0.05$.

5. Discussion

5.1. Behavior

Our study found a significant decrease in the frequency of threatening and fighting behaviors after dietary synbiotic addition. Threatening and fighting are used in groups of birds to establish pecking orders and a dominance hierarchy [20,21]. Agonistic behavior is also used to communicate with others over limited resources like feed and territory [22]. One study found significant differences in the frequency of agonistic behaviors—pecking, fighting, threatening, and chasing—between age, housing system, and genotype [23]. It has been shown in other animals, like germ-free rats [24] and mice [25], that the MGB axis is involved in these complex social behaviors [3]. We hypothesize that the inclusion of the synbiotic was able to modulate negative social interactions like threatening and fighting in laying hens via the MGB axis.

Feather pecking, head pecking, and body pecking were all significantly decreased from supplementation of the synbiotic. One study found that supplementation of β- mannanase and probiotics separately and together had significantly less frequency of aggressive pecks and concluded that dietary probiotics could be an effective strategy to improve welfare [26]. One tenant that is commonly used to study and assess the welfare of animals is the ability to express natural behaviors [27,28]. External factors like management, beak trimming, and housing system can influence laying-hen behavior [29]. Feather pecking is a redirected foraging behavior and may be one way that the laying hen can cope with stressors as well [30,31]. It has been shown in the past with the combination of heavy genetic selection and a barren environment [32], with genetics as a strong influence, as seen in a previous study that found more frequent severe FP and total pecks in a high FP genetic strain [10]. The source of FP behavior may be due to fearfulness or other physiological deficiencies like serotonin and tryptophan [31]. The current poultry industry is transitioning from barren cages to alternate group-housing systems; however, previous studies found continued feather pecking and other agonistic behaviors [33,34]. Influences of agonistic behaviors can be concluded to be multifaceted, and therefore, different strategies to prevent or reduce this behavior must be investigated. Based on our study's results, dietary synbiotic supplementation may have influenced agonistic FP behavior via modulation of the MGB axis and, thus, increased the welfare of laying hens.

5.2. Blood

Our study resulted in a significant decrease in CORT-concentration values at 60 weeks; one study agrees with our finding, with numerically lower levels of CORT in

birds supplemented with a probiotic with *Bacillus subtilis* [9]. Corticosterone has been widely accepted as a marker of stress and a welfare indicator [35]. The primary pathway of the stress response in humans and animals is the hypothalamus–pituitary–adrenal (HPA) axis, and glucocorticoids like CORT are the result of activation of this axis [36]. CORT also provides negative feedback on the HPA axis and stops stimulation of the HPA axis [36]. In the same pathway, circulating ACTH targets the adrenal cortex and stimulates glucocorticoid release [36]. Our results show a significant decrease in circulating ACTH levels in laying hens supplemented with the synbiotic. The microbiota–gut–brain axis can also stimulate the HPA axis and the stress response [37,38]. We hypothesize that the interplay of the gut microbiota and the stimulated synbiotic lowers ACTH and CORT concentrations via the MGB and HPA axes, thus improving the welfare of these laying hens.

In this study, the concentrations of tryptophan were significantly higher in hens from the SYN group compared to the CON group at 60 weeks. One study is in agreement with our findings, as they found an increase of plasma TRP in birds supplemented with a probiotic of *Lactobacillus rhamnosus* [39]. Tryptophan is a precursor to serotonin and has many functions, such as feeding and aggressive behaviors [40,41]. A delicate balance of TRP levels exists, as a deficiency may lead to aggressive behaviors and lower levels of 5-HT. But, excess tryptophan may decrease gentle pecking behavior and CORT levels [40,42,43]. The gut microbiota and tryptophan metabolism have an interconnected relationship, as stress and inflammation in the gut can disrupt TRP metabolism [44]. We hypothesize that the synbiotic positively influenced the gut microbiota and TRP metabolism.

Dopamine levels were significantly lower in laying hens supplemented with a synbiotic at 60 weeks. Dopamine is one neurotransmitter that contributes to cognition and motivation, with the MGB axis playing a role in maintaining DA levels through many interactions [45]. The gut microbiota also contains many species that play a key role with enzymes in the reaction pathway of dopamine [45]. One review found dopamine levels play a role in the frequency of feather pecking [31]. We hypothesize that the impact of the synbiotic on the gut microbiota affects the dopaminergic system via the MGB axis.

The results of the study showed significantly lower levels of plasma 5-HT at 60 weeks of age. Previous studies found a significant increase in circulating 5-HT levels in birds from high feathering pecking genetic lines [10,46]. Enterocromaffin cells in the GIT are responsible for 5-HT storage and release [47]. High stress hormones may be correlated with lower serotonergic energy [48]. In our study, the reverse relationship also exists, with lower CORT levels and high 5-HT levels, supporting our hypothesis of an influence of the serotonergic system via stimulation of the MGB axis. Our study also found a significant increase in 5-HIAA levels in the SYN group at both ages. One study found similar findings to ours, as there was a significant increase in 5-HIAA in HFP laying hens [10]. 5-HIAA is the major metabolite of 5-HT [49]. Our study found an increase in serotonin turnover in birds supplemented with the synbiotic compared to the control at 60 weeks. High and low turnover rates can affect behavior differently. High serotonin turnover is related to depression and panic disorder in humans [50,51]. However, low serotonin turnover can also be related to impulsive violence in humans [52]. 5-HT turnover can reflect the peripheral 5-HT availability, which is in agreement with our results of circulatory 5-HT levels [10].

5.3. Brain Monoamines

The avian brain and its regions have only recently been examined and explored for similarities to the mammalian brain. There is limited research on the connections of the brain to poultry behavior [9,16,53].

5.3.1. Serotonin

Serotonin levels were significantly higher in the hippocampus, while they were significantly lower in the hypothalamus in birds supplemented with the synbiotic. The metabolite of serotonin, 5-HIAA, was significantly higher in the hippocampus but was significantly

lower in the amygdala and hypothalamus in the SYN group. The turnover rate of serotonin was significantly higher in the amygdala but was significantly lower in the hypothalamus and the hippocampus.

Previous studies found no differences in serotonin levels in the hippocampus; however, they were examining different genetic lines with different feather-pecking tendencies [16,18,54]. The hippocampus is mainly in control of spatial awareness [54]. The avian hippocampus may also assist with emotional and cognitive processing like the mammalian hippocampus, where serotonin assists in this regulation [55,56]. Serotonin is synthesized in the brainstem—specifically the raphe nuclei—and then projected out into many regions of the brain including the hippocampus [57]. One study also found similar serotonin genes and receptors in the hippocampus to those of mammals that are related to fear-related behaviors [56,58]. Supplementation of a synbiotic may assist with the modulation of these fear centers via the MGB axis through the regulation of 5-HT and 5-HIAA, but more work is needed.

One study found a significant decrease in 5-HIAA but no differences in the 5-HT levels in the amygdala of birds from an HFP line [16]. The amygdala, which contains the arcopallium, leads to a pathway to the brainstem, is responsible for motor function, and may be involved in anxiety and fear in avian species [59,60]. Serotonin is also important for the regulation of this area in the brain [31]. In humans, low levels of 5-HIAA have been observed in subjects with depression and impulsive tendencies [61]. Because 5-HIAA can also reflect 5-HT bioavailability [10], it seems that synbiotic supplementation may have prevented unnecessary 5-HT from being metabolized to 5-HIAA via inactivation of the serotonergic system through the MGB axis.

The hypothalamus plays a key role in not only welfare status via the HPA axis but also in feeding behavior [62,63]. Weight regulation is especially important for laying hens, as it can negatively affect reproduction and, thus, profit for producers [64]. It also has been observed that intestinal infection can increase both 5-HIAA and 5-HT concentrations in the hypothalamus through serotonergic system stimulation [65]. The interaction of the synbiotic with the MGB axis may have influenced serotonergic system activation and, therefore, lower 5-HT and 5-HIAA levels in the hypothalamus, but further research is needed to validate this.

5.3.2. Dopamine

The levels of dopamine were significantly lower in the hippocampus and amygdala in birds from the SYN group compared to the CON group. One metabolite of dopamine, DOPAC, was significantly lower in the hypothalamus, while another metabolite, HVA, was significantly lower in all three sampled parts of the brain in the SYN group. Dopamine turnover was significantly lower in all three regions of the brain.

Dopamine is a key component to agonistic behavior, especially feather pecking, in laying hens [66]. Dopamine has also been shown to assist with memory formation in humans [67]. Dopamine levels can also reflect the welfare status of laying hens, as it can improve the animal's ability to cope with fear and stress [68,69]. In addition, it has been observed in rats that elevated DOPAC levels may be associated with a stressful environment [70]. One previous study found significantly lower DA levels in the hypothalamus [9]. While our study found lower levels in different regions of the brain, one author suggests that the activity of central noradrenergic neurons is inhibited by serotonergic neurons [9,71]. With similar significant findings between treatments in both serotonergic and dopaminergic molecules, it is safe to assume these two systems are interconnected, with the serotonergic system modulating the dopaminergic systems [72,73].

One previous study found DA turnover to be significantly reduced in the hippocampus and attributed this to fast uptake by dopamine transporters [18,74]. Lower dopamine turnover along with increased CORT may also represent higher stress levels [68], which is the opposite of what we observed in DA turnover levels in the hypothalamus of SYN birds.

This may indicate that supplementation of the synbiotic may increase the physiological welfare status of laying hens.

5.4. Cecal Microbiota

The results of our study found a significant increase in *Lactobacillus* and *Bifidobacterium* spp. counts in laying hens supplemented with the synbiotic. Previous studies agree with our findings of *Bifidobacterium* counts [19,75] while other studies agree with our findings of the *Lactobacillus* counts [76,77]. To reiterate, a synbiotic is essentially a combination of a prebiotic and a probiotic [6], and its supplementation has been shown to change the gut microbiota [78]. Previous studies have used both *Bifidobacterium* and *Lactobacillus* species as probiotics with positive results [79,80]. The supplementation of the synbiotic positively affected the species of these two bacteria, most likely due to the synbiotic containing two *Bifidobacterium* and *Lactobacillus* species. Further research would include further sequencing of the cecal contents.

6. Conclusions

Based on our results, dietary supplementation of a synbiotic blend to late-lay hens exhibits positive findings on physiological measures of behavior, stress, selected brain monoamines, and cecal microbiota counts via modulation of the microbiota–gut–brain axis. Birds in the synbiotic group performed agnostic behaviors less frequently. The dietary synbiotic prolonged lower plasma stress, serotonin, and dopamine measures. Supplementation also showed significant differences in brain monoamines; however, the mechanisms and full pathways need to be elucidated. Cecal microbiota counts of similar bacteria species to the synbiotic were increased. Further sequencing and analysis of specific species are needed. Follow-up studies can include different inclusion rates of the synbiotic, a longer experimental period, and further investigation of the effects of the synbiotic on the gut-microbiota population. Overall, modulation of the microbiota-gut–brain axis via a dietary synbiotic has promising effects on increasing welfare in laying hens.

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