Ingestion of *Lactobacillus rhamnosus* modulates chronic stress-induced feather pecking in chickens

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Feather pecking (FP) is a stress-induced neuropsychological disorder of birds. Intestinal dysbiosis and inflammation are common traits of these disorders. FP is, therefore, proposed to be a behavioral consequence of dysregulated communication between the gut and the brain. Probiotic bacteria are known to favorably modulate the gut microbiome and hence the neurochemical and immune components of the gut-brain axis. Consequently, probiotic supplementation represents a promising new therapeutic to mitigate widespread FP in domestic chickens. We monitored FP, gut microbiota composition, immune markers, and amino acids related to the production of neurochemicals in chickens supplemented with *Lactobacillus rhamnosus* or a placebo. Data demonstrate that, when stressed, the incidence of FP increased significantly; however, *L. rhamnosus* prevented this increase. *L. rhamnosus* supplementation showed a strong immunological effect by increasing the regulatory T cell population of the spleen and the cecal tonsils, in addition to limiting cecal microbiota dysbiosis. Despite minimal changes in aromatic amino acid levels, data suggest that catecholaminergic circuits may be an interesting target for further studies. Overall, our findings provide the first data supporting the use of a single-strain probiotic to reduce stress-induced FP in chickens and promise to improve domestic birds’ welfare.

The mammalian stress response initiates a cascade of physiological and behavioral changes through the neuroendocrine, metabolic, immune, and autonomic nervous systems. When stressors are severe, chronic, multiple, and/or unpredictable, these adaptation processes may fail to return the organism to homeostasis. This failure can lead to chronic disease, which is accompanied by increased hypothalamic–pituitary–adrenal (HPA) axis activity, severe decrease in number and function of lymphocytes T subset profiles, changes to concentrations of immune activation markers, and inflammation-induced changes in catabolism of aromatic amino acids, such as tryptophan (TRP), phenylalanine (PHE) and tyrosine (TYR).

Disturbances in monoaminergic neurotransmitters systems that regulate behavior, in their aromatic amino acids precursors’ metabolism as well as in the HPA-axis such as observed in psychiatric disorders, are concomitant with altered functioning and metabolism of lymphocytes. Lymphocytes are cells of the immune system divided into two major lineages which include the T (thymus-derived) and B (bone-marrow-derived, or bursa of Fabricius–derived in avian species) cells. Subsets of T lymphocytes (distinguished by surface-markers) are responsible for cell-mediated immunity and cytotoxicity but also regulate the inflammatory response and, despite some differences, can be found both in mammals and in chickens.

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In mammals, TRP is largely catabolized into kynurenine (KYN) via multiple enzymes, namely, indoleamine 2,3-dioxygenase 1 and 2 (IDO-1, IDO-2), and tryptophan 2,3-dioxygenase (TDO), with a small percentage being converted into serotonin. PHE is metabolized to TYR to produce the precursor of the catecholamine neurotransmitters dopamine, adrenalin, and noradrenalin. Stress-induced immune activation can dampen the catabolism of these aromatic amino acids into neurochemicals. In turn, in mammals, a pro-inflammatory state decreases serotonin and catecholamine production. Actually, one of the most potent inducers of IDO is the cytokine interferon-γ, produced by T lymphocytes. Similar to mammals, stress can suppress the chicken immune system.

The majority of commercial laying hens hatch and live in standard housing, which tends to be physically, socially, nutritionally, and sensorially restricted environments. These environments can be powerful social and environmental chronic stressors that could induce high rates of severe feather pecking (FP). The gentle form of FP in laying hens targets the tips and edges of feathers without causing damages and is suggested to be similar to social exploration or allo-preening; the severe form of FP is a behavior, whereby individuals grasp, pull and occasionally ingest feathers of their conspecifics. FP can cause feather loss and skin damage, and in some cases, this can escalate to severe injuries and cannibalism. The prevalence of severe FP is reported to range from 15 to 95% on laying hen commercial farms, making it a major animal welfare concern.

Recent research suggests that stress-induced activation of the neuroendocrine system affects gastrointestinal tract function, including gastrointestinal motility and gut microbiota composition. For instance, exposure to stress reduces beneficial microbes, such as *Lactobacillus* species. *Lactobacillus rhamnosus* has been demonstrated to reverse gut dysbiosis.

In this study, we investigated the ability of oral supplementation with a single bacterium to modulate FP behavior, immune biomarkers and T cell phenotypes, as well as cecal microbial composition, the HPA axis and aromatic amino acid metabolism in birds selected for FP and exposed to chronic, repeated, unpredictable stress. To this end, we used *Lactobacillus rhamnosus* JB-1 as an oral supplement as the *Lactobacillus* genus is under-represented in the cecal droppings of feather peckers. Furthermore, *L. rhamnosus* has been demonstrated to attenuate behavioral deficits induced by chronic social stress in mice.

**Results**

*Lactobacillus rhamnosus* supplementation modulates stress-induced pecking behavior and feather cover. The sequence of stressors in the present study triggered severe feather pecking (FP) in birds supplemented with *L. rhamnosus* (Lacto) or with drinking water as a control (Placebo) (Figure 1a). As expected, this was accompanied by a significant increase in the damage to the feather cover (OR = 4.10, 95% CI 1.14–14.78, F_{1,38} = 4.78, P = 0.032).

Interestingly, the Lacto treatment significantly attenuated severe FP behavior in the presence of stress by 2.5-fold relative to birds receiving the Placebo (Figure 1a). Indeed, the incidence of severe FP (P = 0.664) and the feather cover score (OR = 1.15, 95% CI 0.31–4.25, P = 0.996) in stressed, Lacto birds was not significantly different from that of non-stressed Lacto birds. In contrast, stressed, Placebo supplemented birds performed a significantly higher number of severe FP events (P = 0.032, Figure 1a) and displayed a tendency to have a more deteriorated feather cover than their non-stressed counterparts (OR = 14.54, 95% CI 1.59–132.70, P = 0.084). It is also noteworthy that Lacto birds performed gentle FP more frequently than Placebo birds (F_{1,6} = 11.94, P = 0.014; Figure 1b), regardless of the stress treatment.

*Lactobacillus rhamnosus* treatment prevents stress-induced alterations to the cecal microbiota. The species richness of the microbial communities (i.e., alpha diversity) was measured using the Shannon’s diversity index. We found that alpha diversity was similar between the test groups and was not influenced by either Lacto supplementation (F_{1,10} = 0.380, P = 0.539) or stress (F_{1,10} = 0.425, P = 0.516; Figure 2). Changes in relative abundance of individual operational taxonomic units are shown in Supplementary Figure S1.

Beta diversity accounts for differences in the bacterial community composition. In contrast to alpha diversity, beta diversity measured using the Bray–Curtis dissimilarity, was significantly altered by chronic, unpredictable stressors in the Placebo birds (median: NS-Placebo: 0.594, S-Placebo: 0.619; P < 0.05; Figure 3). Stressed Placebo...
birds also had a higher dissimilarity value relative to the baseline (18 weeks of age [woa]) than stressed Lacto supplemented birds (median: S-Placebo: 0.619, S-Lacto: 0.590; P < 0.05; Fig. 3).

**Lactobacillus rhamnosus** supplementation regulates chronic stress-induced alterations of T-regulatory cells population in the spleen and cecal tonsils. The common T cell marker, CD3 was used in combination with other cell surface markers to identify changes to T cell sub-populations in response to *L. rhamnosus* supplementation and the sequence of stressors introduced in this experiment. An example of the gating strategy is shown in Supplementary Figure S2. According to flow cytometry analysis, neither Lacto supplementation nor stress impacted the proportion of T helper cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+) in the spleen (Fig. 4a,b) or in the cecal tonsils (Fig. 4d,e).

In contrast, we report a significant increase of the proportion of regulatory T cells (Treg; CD4+CD25+) in the cecal tonsils (P < 0.001, Fig. 4f) and a tendency of increase in the spleen (P = 0.088, Fig. 4c) in stressed Lacto birds compared to stressed Placebo birds. Indeed, Treg cells represented 47.6% of the cecal tonsil T cell population in the former group, while this proportion was merely 28% in the latter (F3,3 = 2.18, P < 0.001). In non-stressed birds, splenic Treg cells were also significantly more abundant in Lacto birds compared to Placebo birds (11.75% vs. 5.0% F3,3 = 12.79, P = 0.042). This difference in the spleen was further increased when Lacto birds were stressed (F3,3 = 33.61, P = 0.014). In the absence of stress, the proportion of Treg cells in the cecal tonsils were similar between groups.

**Severe FP phenotype is associated with elevated PHE levels.** Stressors, *L. rhamnosus* supplementation and their interaction did not significantly change peripheral plasma levels of TRP, PHE, TYR, KYN, and

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**Figure 1.** Expression of severe and gentle feather pecking behavior. Frequency of severe (a) and gentle (b) feather pecks per minute (Least Squares Means ± Standard Error) between weeks 24 and 26 based on supplementation (Lacto: *L. rhamnosus*, Placebo: Placebo supplementation) and stress (S: stressed, NS: non-stressed) treatment (n: S-Placebo = 22, NS-Placebo = 22, S-Lacto = 20, NS-Lacto = 22). Asterisks (*) indicate statistically significant differences (P < 0.05).

**Figure 2.** Alpha diversity of microbial communities of cecal samples from laying hens. Alpha diversity of microbial communities of cecal samples measured using the Shannon index from laying hens at 27 weeks of age based on supplementation (Lacto: *L. rhamnosus*, Placebo: Placebo supplementation) and stress (S: stressed, NS: non-stressed) treatment (n: S-Placebo = 22, NS-Placebo = 22, S-Lacto = 18, NS-Lacto = 22).
Figure 3. Beta diversity of microbial community diversity of cecal samples from laying hens. Beta diversity analysis as shown by Bray–Curtis distance calculations. The dissimilarity values for each experimental group based on supplementation (Lacto: *L. rhamnosus*, Placebo: Placebo supplementation) and stress (S: stressed, NS: non-stressed) treatment compare week 18 (baseline) and week 27 (n: S-Placebo = 22, NS-Placebo = 22, S-Lacto = 18, NS-Lacto = 22). Asterisks (*) indicate statistically significant differences (P < 0.05).

Figure 4. T cell sub-populations in the spleen and cecal tonsils. T cell sub-populations in the spleen (a–c) and cecal tonsils (d–f) after three weeks of *L. rhamnosus* (Lacto) or placebo (Placebo) supplementation and stress treatment (S: stressed, NS: non-stressed). Sub-populations were identified using the following combinations of cell surface markers: T helper cells = CD3+ CD4+; cytotoxic T cells = CD3+ CD8+; T regulatory cells = CD4+ CD25+ (n: S-Placebo = 4, NS-Placebo = 4, S-Lacto = 4, NS-Lacto = 4). Asterisks (*) indicate statistically significant differences (P < 0.05).
their relevant ratios. Furthermore, the TRP:(PHE + TYR) ratio, as well as the nitrite, neopterin and corticosterone (CORT) concentrations were similar between groups (Table 1).

However, birds exhibiting a severe FP phenotype, characterized as individuals that displayed at least once severe FP bout between 24–26 woa, had higher peripheral plasma PHE levels (F1,77 = 7.88, P = 0.006) and a tendency for higher TYR levels (F1,77 = 3.84, P = 0.054) compared to birds classified as non-feather peckers (Table 2).

**Discussion**

Recent advances in feather pecking (FP) research in laying hens explore the role of bidirectional communication between the gut and the brain and have suggested that severe FP may be linked to neurobiological dysfunction35. Fundamental work has shown that the gut microbiota and the microbiota-derived metabolites are distinct in feather peckers versus non-peckers, whereby the former has a significantly lower abundance of *Lactobacillus* bacteria in the ceca47–49. In murine models, specific *Lactobacillus* gut bacteria can modulate central nervous system-driven behaviors50,52, alter the monoaminergic system53 and their amino acid precursors54,55, impact the hypothalamic–pituitary–adrenal (HPA) axis50, and influence the immune system52,56–58. Interestingly, these same pathways are associated with severe FP in laying hens24,41,43,45,46,59–61. Nevertheless, the ability of a single bacterial strain to prevent or reduce FP and/or impact the associated physiological systems has not yet been investigated.

Therefore, the aim of the present study was to determine whether oral supplementation with a single *Lactobacillus rhamnosus* strain could modulate stress-induced FP behavior and associated physiological parameters in laying hens that are genetically selected for high FP activity. To this end, we report the effect of stress and *L. rhamnosus* supplementation on FP, T cell phenotypes, the cecal microbiota, corticosterone levels, and the metabolism of the aromatic amino acids tryptophan (TRP), phenylalanine (PHE), and tyrosine (TYR) (Fig. 5).

The sequences of stressors administered to the experimental subjects of this study was designed to mimic the chronic, unpredictable, social and non-social environments that laying hens encounter in commercial settings. The FP data (Fig. 1a) mirror the literature, showing that severe FP increased in response to stress 24. A critical finding of this study is an oral *L. rhamnosus* supplement (Lacto group) prevented the stress-induced increase in FP.

Table 1. Least Squares Means (± Standard Error) of amino acid, immune biomarker and corticosterone concentrations in laying hens (n: S-Placebo = 22, NS-Placebo = 22, S-Lacto = 20, NS-Lacto = 22) after 8 weeks of treatment based on supplementation (Lacto: *L. rhamnosus*, Placebo: Placebo supplementation) and stress (stressed, non-stressed). No statistically significant difference was found due to the stress treatment, *L. rhamnosus* supplementation or their interaction (P-value shown refers to the interaction).

|                      | Lacto (n = 42) | Placebo (n = 44) | P-value |
|----------------------|---------------|-----------------|---------|
|                      | Stressed (n = 20) | Non-Stressed (n = 22) | Stressed (n = 22) | Non-Stressed (n = 22) |
| Tryptophan (TRP) (µmol/L) | 73 ± 2.3 | 71 ± 2.1 | 72 ± 2.1 | 75 ± 2.1 | 0.2914 |
| Tyrosine (TYR) (µmol/L) | 93 ± 4.6 | 94 ± 4.6 | 93 ± 4.3 | 96 ± 4.6 | 0.7874 |
| Phenylalanine (PHE) (µmol/L) | 90 ± 3.7 | 90 ± 3.6 | 91 ± 3.3 | 92 ± 3.6 | 0.8802 |
| TRP:(PHE + TYR) | 0.40 ± 0.019 | 0.40 ± 0.019 | 0.40 ± 0.017 | 0.41 ± 0.019 | 0.9692 |
| KYN:TRP (µmol/mmol) | 3.0 ± 0.32 | 3.9 ± 0.60 | 3.2 ± 0.41 | 2.8 ± 0.30 | 0.1119 |
| PHE:TYR (µmol/µmol) | 1.00 ± 0.032 | 0.98 ± 0.033 | 0.96 ± 0.030 | 0.97 ± 0.032 | 0.8376 |
| Kynurenic acid (KYN) (µmol/L) | 0.22 ± 0.021 | 0.29 ± 0.040 | 0.21 ± 0.024 | 0.22 ± 0.021 | 0.3222 |
| Nitrite (µmol/L) | 53 ± 9.8 | 37 ± 6.5 | 50 ± 10.8 | 49 ± 8.7 | 0.3843 |
| Neopterin (nmol/L) | 2.6 ± 0.11 | 2.55 ± 0.089 | 2.63 ± 0.095 | 2.60 ± 0.087 | 0.7889 |
| Corticosterone (pg/mL) | 2093 ± 538.1 | 1644 ± 423.6 | 1451 ± 359.0 | 1270 ± 308.9 | 0.8277 |

Table 2. Least squares means (± Standard Error) of the amino acid, immune biomarker and corticosterone concentrations in laying hens according to their feather pecking phenotype. A feather pecker was characterized as a bird that displayed severe feather-pecking behavior at least once between 24–26 weeks of age.

|                      | Feather pecker (n = 30) | Non-feather pecker (n = 56) | P-value |
|----------------------|------------------------|-----------------------------|---------|
| Tryptophan (TRP) (µmol/L) | 74 ± 1.8 | 72 ± 1.3 | 0.2444 |
| Tyrosine (TYR) (µmol/L) | 100 ± 3.6 | 91 ± 2.7 | 0.0537 |
| Phenylalanine (PHE) (µmol/L) | 97 ± 2.8 | 87 ± 2.1 | 0.0063 |
| TRP:(PHE + TYR) | 0.59 ± 0.015 | 0.41 ± 0.011 | 0.2723 |
| KYN:TRP (µmol/mmol) | 3.1 ± 0.26 | 3.1 ± 0.21 | 0.8416 |
| PHE:TYR (µmol/µmol) | 0.99 ± 0.026 | 0.98 ± 0.019 | 0.7619 |
| Kynurenic acid (KYN) (µmol/L) | 0.24 ± 0.020 | 0.23 ± 0.015 | 0.7218 |
| Nitrite (µmol/L) | 50 ± 7.5 | 45 ± 4.6 | 0.6043 |
| Neopterin (nmol/L) | 2.53 ± 0.077 | 2.68 ± 0.059 | 0.1370 |
| Corticosterone (pg/mL) | 2180 ± 528.2 | 2278 ± 398.3 | 0.8842 |
Our results suggest that a single bacterial strain is thus protective against social behavioral deficits triggered by chronic, unpredictable environments in laying hens consistent with data from studies using murine models. In addition to its ability to prevent severe FP, the beneficial effect of an L. rhamnosus supplement is further evidenced by the increase of gentle FP in Lacto birds (Fig. 1b). Gentle FP has been suggested to be a form of explorative, pro-social pecking that plays an important role in the building and maintenance of positive, social relationships among chicks. Given the divergent motivations behind gentle and severe FP, it is critical that studies investigating FP differentiate these two forms. In mice, L. rhamnosus likewise mediates pro-social behavior which is accompanied by changes in cerebral GABAergic activity via the vagus nerve. A recent study showed that L. rhamnosus interacts with the enteric nervous system (ENS) in laying hens. Considering that the vagus nerve connects the ENS with the central nervous system, the ENS may indeed be an important component of the pathway through which L. rhamnosus promotes sociable, gentle FP behavior in chickens. Further research is required to test this assumption.

While oral supplementation focuses on the microbiota-gut-brain axis, it is important to acknowledge the potential impact of this treatment on the oral microbiota and its possible consequences on FP behavior. Indeed, the transfer of the oral microbiota to the feather cover via preening or pecking may change the olfactory features or taste of feathers. Enteric and environmental bacteria influence olfaction in mice, alter body odor in birds, and are responsible for recognition of species. Thus, it is important to acknowledge that, in the present study, oral supplementation of L. rhamnosus may have resulted in the transfer of the bacteria to the feather cover via preening or pecking leading to a change in the olfactory features or taste of feathers. This is turn, may have promoted gentle FP and dissuaded birds from performing severe FP. While the theory needs to be tested
experimentally, this association may identify the feather cover or skin of the birds as a significant contributor to social behavior.

In addition to reducing severe FP and improving feather cover, our data show that the stress treatment significantly altered the microbiota profile of the cecal droppings. The stressed Placebo hens had a higher increase in dissimilarity from 18 to 27 weeks compared to the non-stressed Placebo hens (Fig. 3). However, the stress treatment did not alter the changes in the microbiota profile between 18 and 27 weeks in Lacto hens (Fig. 3). Additionally, the stressed-Lacto birds displayed significantly lower dissimilarity in their cecal microbial community diversity from week 18 to 27 than the stressed-Placebo. There were also OTU level differences between stressed Placebo hens and Lacto hens (Figure S1). This suggests that L. rhamnosus supplementation is preventing stress-induced disruption of cecal bacterial community structure in laying hens. However, regardless of the bird's supplementation and stress treatments, no significant correlations were found between the Shannon Index values and the bird's gentle and severe pecking phenotype (data not shown). It should be noted that we only assessed the effect of Lacto treatment under stressful condition on microbiome composition. Indeed, previous studies have demonstrated that L. rhamnosus shows behavioral effects under challenging, i.e., stressful situations and does so without altering microbiota composition22. Furthermore, it has been observed that the L. rhamnosus can induce changes within minutes on the gut motility ex-vivo in mice48 and in laying hens49, further suggesting that the effect of Lactobacillus is mediated through other avenues than microbiota composition changes. Thus, the effects of supplementation alone on microbiome would provide no mechanistic insight. The actual mode of action of L. rhamnosus is yet to be shown experimentally.

The immunomodulatory capacities of L. rhamnosus are well recognized in mammals57,69,70. We presently report that the L. rhamnosus supplement also impacts immune measurements in birds. In particular, L. rhamnosus supplementation significantly increased the regulatory T (Treg) cell population in the cecal tonsils and the spleen in response to stress (Fig. 3). Regulatory T cells crucial for regulating the immunological response and preventing autoimmunity71. Thus, we conclude that the bacterium was able to influence the local immune system of the hens similar to previous observations in mice57,69. Chicken cecal tonsil cells respond more rapidly than spleen cells to the bacterial stimuli, which could explain differences between these tissue sites71. Short-chain fatty acids (SCFAs) produced by commensal organisms are known to trigger the increase of Treg in mammals and chicken cecal tonsils72–74. The ability of L. rhamnosus to produce SCFAs that mediate immune activation is yet to be investigated, and further research should consider testing for SCFAs to help identify the modes of action of the bacteria.

Nitric oxide (NO) is produced as part of the innate, pro-inflammatory immune response when nitric oxide synthase production is stimulated by IFN-γ. Kujundžić and Lowenthal52 demonstrated that the expression of inducible nitric oxide synthase (iNOS) and NO formation can be efficiently stimulated by chicken IFN-γ in the chicken macrophage cell line HD11, reaching nitrite concentration of approximately 40 μmol/L after 24 h of treatment. Since NO is rapidly converted to the more stable metabolite nitrite, the latter is used as a surrogate marker of NO53. We report no statistically significant differences in the nitrite levels between treatment groups. However, under non-stressed conditions, the Lacto birds had a lower plasma nitrite concentration compared to the Placebo group. Moreover, in parallel to increasing Treg cells, stress caused an increase in nitrite levels in Lacto supplemented birds. Taken together, this set of data suggests that the baseline inflammation status is lower in Lacto-supplemented birds. The overall increase of the Treg population in stressed Lacto birds may be an attempt to counteract the induced immune activation77. Thus, the pro-inflammatory response leading to more nitrate may be balanced out by the anti-inflammatory effect of Treg cells in the long term.

Corticosterone (CORT) is a surrogate measure of the HPA axis activation and is used as an indicator of acute stress in birds42,78. Surprisingly, we observed no increase in the plasma CORT concentration under stress. Nevertheless, it is noteworthy that CORT levels recorded in this study were low compared to a recent study using the same genetic line49 and similar to those in resting birds40. We further report that CORT measurements between peckers and non-peckers were similar, confirming previous observations41. Despite being an indicator of acute stress, chronic stress is known to downregulate CORT production through a negative feedback loop79. Therefore, it is possible that the sequence of stressors used in the study dampened CORT levels over time, as this stress regimen was intended to represent chronic stress. In addition, it is important to acknowledge that only two timepoints were used to measure plasma CORT levels, which may not have adequately represented changes in CORT concentrations under chronic stress conditions.

Neither the ingestion of L. rhamnosus nor the chronic use of stressors significantly altered TRP, PHE, and TYR levels, their ratios, or the downstream metabolite, KYN. In humans, an elevated KYN:TRP ratio is an indicator of TRP degradation by the IDO-1 enzyme activated during acute innate and adaptive immune responses7,18–20. However, this effect of IDO-1 activation is not observed in laying hens and Lacto hens (Figure S1). This suggests that L. rhamnosus supplementation is preventing stress-induced disruption of cecal bacterial community structure in laying hens. However, regardless of the bird's supplementation and stress treatments, no significant correlations were found between the Shannon Index values and the bird's gentle and severe pecking phenotype (data not shown). It should be noted that we only assessed the effect of Lacto treatment under stressful condition on microbiome composition. Indeed, previous studies have demonstrated that L. rhamnosus shows behavioral effects under challenging, i.e., stressful situations and does so without altering microbiota composition22. Furthermore, it has been observed that the L. rhamnosus can induce changes within minutes on the gut motility ex-vivo in mice48 and in laying hens49, further suggesting that the effect of Lactobacillus is mediated through other avenues than microbiota composition changes. Thus, the effects of supplementation alone on microbiome would provide no mechanistic insight. The actual mode of action of L. rhamnosus is yet to be shown experimentally.

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Neither the ingestion of L. rhamnosus nor the chronic use of stressors significantly altered TRP, PHE, and TYR levels, their ratios, or the downstream metabolite, KYN. In humans, an elevated KYN:TRP ratio is an indicator of TRP degradation by the IDO-1 enzyme activated during acute innate and adaptive immune responses7,18–20, linking immune activation to the development of neuropsychiatric symptoms7. Actually, one of the most potent inducers of IDO is IFN-γ, produced by T cells22, while certain IDO-expressing cells inhibit T cell activation20,81. In parallel, pro-inflammatory cascades are also associated with increased phenylalanine hydroxylase (PAH) activity23, which metabolizes PHE to TYR and is reflected by the PHE/TYR ratio. In chronic episodes of immune activation, PHE and PHE/TYR ratio are elevated at the expense of dopamine82, a neurotransmitter which has actions on both the nervous and the immune systems43 and is involved in gut-brain signaling84. Further down the PHE-TYR-Dopamine pathway, norepinephrine differentially regulates naïve and effector CD8+ T cell activity85 and decreases functionality and proliferation of CD8+ T cell86. Thus, in mammals, aromatic amino acid metabolism and the monoaminergic neurotransmitter systems are associated and connected to the host peripheral immune system responsiveness. The divergence from the mammal system is likely due to evolutionary differences in laying hen's amino acid metabolism, notably the lack of IDO-187,88. IDO-2, the isoenzyme, has a relatively low affinity for TRP and a low enzymatic efficiency because of its very low catalytic velocity87. So far, no direct in vivo evidence suggests that the induction of aromatic amino acid metabolism in laying hens is mediated via immunological cascades. Interestingly, though Kujundžić and Lowenthal52 reported the upregulation of TRP
degrading enzymatic activity in IFN-γ stimulated lysates of the chicken macrophage cell line HD11 that is able to produce KYN. Nevertheless, the KYN concentrations of these lysates decreased over a period of 24 h after treatment. It is possible that the activity of other downstream enzymes in the KYN breakdown pathway may be responsible for the consumption of KYN. Therefore, it remains unclear whether cytokines can stimulate TRP catalysis in chicken macrophages.

As this study focuses on laying hens and attempts to mimic commercial settings, it is important to emphasize that data collection coincided with the onset of egg-laying. The nutritional requirements of hens undergo a significant shift during this period, and resources are preferentially channeled towards protein synthesis for egg formation, while requirements for growth are minimal. Additionally, the pullets bred for high FP behavior may not have the same requirement as commercial birds. Thus, we also hypothesize that the respective amino acids concentrations in the commercial diet we used may not have been sufficient for the young laying hens in this study. Indeed, we observed a 14% decrease in plasma TRP and TYR levels between 18 and 26 weeks of age. It is, therefore, possible that the physiological stress encountered by hens during this transitional period may have masked the true changes to plasma amino acid concentrations in response to stress exposure. Finally, there is also evidence suggesting that plasma amino acid levels vary depending on the timing of blood extraction and the amount of feeding. As such, one limitation of this study is that fasting levels of TRP, PHE, and TYR were not recorded.

Yet another important, social context that must be considered, is that the birds in the present study were familiarized with human-interactions which may have attenuated the impact of stressors. Furthermore, social disruptions happened concurrently with the replacement of the bedding material. Fresh bedding material is an environmental enrichment, which, in turn, may have countered the intended stress of the social disruption.

We found that birds that exhibited a severe FP phenotype had significantly higher PHE and a tendency for higher TYR levels than non-peckers (Table 2). However, we report no difference in PHE:TYR ratio between peckers and non-peckers, suggesting that the relative change in PHE and TYR was equal. PHE and TYR can cross the blood–brain barrier. In mammals, the plasma levels are good indicators of PHE and TYR brain levels. Peckers may have higher central levels of PHE and TYR response to stress, producing higher levels of catecholamines, which may indicate a more sensitive and hyperactive response to stress. Thus, our results are in line with the higher dopaminergic activity found in chickens exhibiting severe FP and injurious pecking. This work reinforces the theory that the dopamine pathway plays a role in stress regulation in chickens. However, chickens have different metabolic properties and requirements for amino acids compared with mammals. These results should be considered with caution as the correlation between central and peripheral levels in poultry species is yet to be tested.

To the authors’ knowledge, we show for the first time that administration of L. rhamnosus in laying hens can alter brain and immune function under stress, resulting in reduced severe FP and improved feather cover in birds. The gut microbiota was also significantly more resistant to stress-induced changes when birds received the Lactobacillus rhamnosus supplementation (Fig. 3). Furthermore, the data presented herein shed light on the potential associations between the immune system, the gut microbiome, and neuro-chemical pathways that contribute to FP.

In conclusion, our data suggest that single-microbe supplementation can ameliorate severe FP in laying hens. Further research should elucidate whether L. rhamnosus drives the changes to FP behavior (i.e., direct effect), or whether the bacterium modulates the diversity and/or function of other microbial communities, metabolites, and the immune system, thereby triggering other behavior that result in the observed FP shifts (i.e., indirect effect). Additionally, results would need to be replicated in other genetic lines, as well as commercial lines. The feasibility of supplementation under commercial conditions should also be investigated. Our findings identify potential biological mechanisms behind the therapeutic effects of probiotics which paves the way for individualized, microbial interventions in millions of domestic birds with a history of FP.

Methods

Ethical statement. The experiment was approved by the Animal Care Committee at the University of Guelph (Animal Utilization Protocol #3206). The study was carried out in accordance with relevant guidelines and regulations as well as the ARRIVE guidelines.

Animals and housing conditions. Non-beak-trimmed White Leghorn laying hens, originating from a selection experiment in which birds were divergently selected for high (HFP) and low (LFP) feather pecking behavior, were used in this study. A total of 86 HFP birds were wing tagged at hatch and housed in 4 floor pens (22 ± 5 birds) until 14 weeks of age (woa) under conventional management conditions at the Research Station of the University of Guelph, Guelph, Ontario, Canada. At 14 woa, birds were randomly allocated to 12 identical floor pens (7 ± 1 birds). Each pen (1.6 m2) was littered with wood shavings as bedding material and equipped with one round metal feeder (43 Ø cm), a 4 nipple drinker, two nest boxes, 1 platform (125 × 31 cm, 90 cm above the ground) and two elevated perches (15 cm of perch/ hen; 55 and 120 cm above the ground). One camera (Samsung SNO-5080R, IR, Samsung Techwin Co., Gyeongi-do Korea) was ceiling-mounted above each pen to allow for a full view of the pen. Auditory contact and smell were allowed between pens, but visual contact was prevented by opaque PVC boards. Light was provided at 20 lx from 05:00 until 19:00. The average daily temperature was 20 ± 0.5 °C. Birds had ad libitum access to well water and commercial layer feed.

Lactobacillus rhamnosus supplementation and chronic, unpredictable stress treatments. An overview of the experimental timeline is represented in Fig. 6. Half of the pens were systematically assigned to receive either an oral supplementation with 5 × 108 Lactobacillus rhamnosus JB-5 (Lacto, n = 6 pens, 42 birds) resuspended in 1 mL of drinking water or a placebo of 1 mL drinking water (Placebo, n = 6 pens, 44 birds). Lacto-
bacillus rhamnosus JB-1 was a gift from Alimentary Health Inc., Cork, Ireland to McMaster University. Between 19 to 26 woa birds were supplemented individually using 12 mL plastic syringes. Birds received treatments once a day, Monday to Friday mornings, between 9:00 and 10:30. To limit additional stress due to daily handlings, birds were trained, prior to the experiment, to enter a dog crate (93 × 61 × 58 cm) and receive supplementations using canned sweet whole kernel corn as a food reward.

Following 5 weeks of Lacto or Placebo supplementation, three pens from each group were randomly assigned to undergo a sequence of stressors (S, n = 6 pens, 42 birds) spanning a period of 3 weeks (Fig. 6). The remaining pens continued to receive Lacto or Placebo supplements in the absence of stress (NS, n = 6 pens, 44 birds). The five stressors used in this experiment were: social disruption (5 times), physical restraint of all individuals in a pen for 1 h in a transport crate (4 times), individual manual restraint for 5 min on a table outside of the experimental room (4 times), blocking nest boxes used for egg-laying for 4 days (twice), and blocking perches used for roosting for 4 days (twice). The 3-weeks stress regimen was designed to mimic the unpredictable and repeated stressors that hens encounter in commercial farm settings. These were reported as potential triggers for SFP in the literature. The social disruption stressor consisted of splitting the pen into two subgroups of 3 to 4 individuals and mixing them with another subgroup from a different stressed pen that had received the same supplement (Lacto or Placebo). These newly mixed, socially disrupted, groups of birds were moved into new, identical pens with fresh bedding material.

Behavioral observations and physical examination. Birds were individually identified using numbered silicone backpacks (8 × 6 × 0.5 cm) provided 2 weeks before the experiment started. Backpacks were fastened around the wings via two elastic straps secured to the backpacks with metal eyelets. The time windows used to observe FP activity were determined by pilot observations. Baseline behavioral observations were recorded over three days during week 18 (5 min in the morning at 8:00 and 5 min in the afternoon at 17:00). During the simultaneous supplementation and stress treatments (weeks 24–26), birds were observed for 5 min at 8:00 and 10 min post-stressors. In total, each individual bird was observed for 185 min: 11 morning observations prior to supplementation treatment (5 min each) and 13 post-stress observations (10 min each).

All-occurrence sampling was used to record initiators and recipients of gentle and severe FP interactions. An occurrence was defined as a sequence of uninterrupted behavior lasting more than 4 s aimed at the same bird. Gentle FP was defined as gentle peck(s) at the tips and edges of feathers of conspecifics without their removal. Severe FP was defined as intended, forceful peck(s) towards the feathers/body (not the head) of conspecifics that may remove feathers and cause injury. All behavioral observations were performed by a trained, blinded observer.

Physical examinations to determine feather cover and bodyweight of each bird was performed during weeks 18 and 27 (Fig. 6). Feather cover was assessed using a severity scale from 0 (no or slight wear, nearly intact feathering) to 2 (at least one featherless area ≥ $2 Canadian coin) on the neck, back or tail.

Blood collection and analysis. Blood samples were collected (3.5 mL/hen) at 18 and 27 woa from the wing vein using EDTA-coated vacutainer tubes. Individual birds were sampled, one hour post-feeding, on the same day of the week and at the same time of day (between 10:00 and 14:00) during both sampling weeks. After collection, samples were stored on ice (maximum of 4 h) until centrifugation (4 °C, 2,500 rpm, 15 min) for plasma separation. Plasma was aliquoted into 1.5 mL microtubes and stored at –80 °C until further analysis.

The concentration of amino acids and their derivatives, nitrite and neopterin were determined performed as reported previously. Briefly, samples were analysed via reversed-phase HPLC using a LiChrosorb C18.
The cecal samples were transferred into sterile 1.5 ml Eppendorf tubes and stored at –80 °C until further processing of the sample. After a minimum of 24 h, one gram of cecal feces material per bird was sampled using a clean spatula. Cecal excreta were identified by its characteristic homogeneous, smooth and creamy texture and dark colour. Samples were taken from the middle of the cecal discharge to avoid contamination with non-cecal material. Excreta from each bird were transferred to individual cages (45.7 L × 45.7 W × 40.5 H cm) for a maximum of 48 h. The hens had visual contact but no physical access to their neighbors. Clean foil trays were placed underneath each cage to collect droppings from each bird. After a minimum of 24 h, one gram of cecal feces material per bird was sampled using a clean spatula. Cecal excreta were identified by its characteristic homogeneous, smooth and creamy texture and dark colour. Samples were taken from the middle of the cecal discharge to avoid contamination with non-cecal excreta. The cecal samples were transferred into sterile 1.5 ml Eppendorf tubes and stored at –80 °C until further processing of the sample.

DNA extraction was carried out as previously described30,52, using a modified protocol to increase recovery of bacteria across taxa107. 16S rRNA sequencing was carried out using a modified, barcoded Illumina sequencing method109. The 341F and 518R primers were used to amplify 16S rRNA from cecal excreta samples. A MiSeq Illumina sequencer in the McMaster Genome Center was used for paired-end reads of the V3 region109 and the intra- and inter-assay coefficients of variation based on controls were 1.5% and 6.5% for low quality data110. Abundant Operational Taxonomic Units (OTUs) were used to produce clustered sequences of OTUs111, sequencing produced 5.960 OTUs, and a minimum, maximum, and median of 8,856, 126,407, and 56,768 reads/sample respectively. Data were rarefied at a sequencing depth of 8,856 reads/sample using QIIME114. The Shannon diversity index was calculated for alpha-diversity analysis, and Jackknife resampling was used to estimate TRP metabolism along the KYN axis. In humans, this ratio is used as an index of the cellular immune system106. PHE:TYR ratios were calculated as phenylalanine 4-hydroxylase (PAH) activity, which converts PHE to TYR105. TRP:(PHE + TYR) is a substitution for the commonly used ratio of TRP to the large neutral amino acids. As described in Wurtman et al.106, this ratio indicates the competition of TRP with other amino acids for uptake across the blood–brain-barrier. However, these findings relate to mammals and may not be fully translatable to poultry because of possible evolutionary variations107,108, and thus, the results should be approached with caution.

To estimate nitric oxide (NO) production, the stable NO metabolite nitrite was measured in the plasma sample collected using a modified Griess assay (Merck KGaA, Darmstadt, Germany). Neopterin levels were measured by enzyme-linked immunosorbent assay (ELISA) with a detection limit of 2.0 nmol/L (BRAHMS Diagnostics, Hennigsdorf, Germany). Corticosterone concentrations were analysed using an ELISA kit (Enzo Life Sciences Inc., NY, USA). The intra- and inter-assay coefficients of variation based on controls were 1.5% and 6.5% for low quality controls, 0.9% and 2.5% for medium quality controls, and 0.9% and 1.4% for high quality controls, respectively.

**Sampling of cecal droppings and 16S rRNA sequencing.** During weeks 18 and 27, birds were transferred to individual cages (45.7 L × 45.7 W × 40.5 H cm) for a maximum of 48 h. The hens had visual contact but no physical access to their neighbors. Clean foil trays were placed underneath each cage to collect droppings from each bird. After a minimum of 24 h, one gram of cecal feces material per bird was sampled using a clean spatula. Cecal excreta were identified by its characteristic homogeneous, smooth and creamy texture and dark colour. Samples were taken from the middle of the cecal discharge to avoid contamination with non-cecal excreta. The cecal samples were transferred into sterile 1.5 ml Eppendorf tubes and stored at –80 °C until further processing of the sample.

**Spleen and cecal tonsil samplings and analysis.** At week 28, 16 hens (4 Lacto-S; 4 Lacto-NS; 4 Placebo-S; 4 Placebo-NS, whereby S = stressed and NS = non-stressed) were killed by cervical dislocation. One cecal tonsil and the spleen were harvested from each bird within 3 min after death and kept in 5 mL of 5% Fetal Bovine Serum (FBS) containing RPMI medium. Spleen cells were isolated by scratching the spleen on a 40 μm cells strainer with a sterile syringe nozzle using sufficient pressure to completely crush the full spleen on a 6 well plate containing 6 mL cold Phosphate-buffered saline (PBS). Cell suspensions were then transferred into 15-mL falcon tubes and plates were rinsed with an additional 6 mL of cold PBS and pooled into the tubes. Cell suspensions were then centrifuged at 1,500 rpm at 4 °C for 10 min. Cells were then resuspended in red blood cell (RBC) lysis buffer for 3–4 min and washed with cold PBS at 1500 rpm for 10 min at 4 °C. Cells were resuspended in 5% FBS-RPMI medium. Cecal tonsils were cut into small pieces and digested in 2 mL of 3% FBS-RPMI containing 5 μM EDTA and 20 μg/mL Collagenase IV (Sigma Aldrich, Oakville, Canada) at 37 °C with shaking for 40 min. Then, cecal suspensions were passed through 40 μm strainer and washed with 5% FBS-RPMI medium by centrifuging at 1,500 rpm (4 °C) for 10 min. Cells were then resuspended in 5% FBS-RPMI medium. Viable spleen and cecal tonsil cells were counted by Trypan Blue exclusion and diluted in 100 μl of Fluorescence-activated cell sorting (FACS) buffer (PBS + 2% FBS) to a concentration of 10^6 cells/ml. Both splenocytes and cecal tonsillar cells were stained for regulatory T cells (Treg) markers using the following antibodies conjugated to their respective dyes: Mouse Anti-Chicken CD3—APC, Mouse Anti-Chicken CD4—PE, Mouse Anti-Chicken CD8a—PerCP Cy5.5 (Southern Biotech, Birmingham, AL, USA) and Human Anti-Chicken CD25—FITC (BIO-RAD, Mississauga, ON Canada).
Data were acquired using FACS Celesta (Becton Dickinson, Oakville, ON, Canada) and analysed using FlowJo (TreeStar, Ashland, OR, USA).

**Statistical analysis.** FP frequencies were determined per individual per min and averaged at the pen level. Feather cover was scored across different body regions and these scores were combined to determine presence (at least one score > 0) or absence (all regions scored 0) of plumage damage.

The SAS software (version 9.4, SAS Institute, Cary NC) was used for all statistical computations except for the microbiota and immunological data, which were analyzed with GraphPad Prism 6 and SPSS using a two-tailed Student’s t-test, Mann–Whitney U-test, Kruskal–Wallis test, or ANOVAs, with Dunn’s multiple comparisons post-hoc tests. The assumptions of normally distributed residuals and homogeneity of variance were examined graphically with the use of QQ plots. Data was transformed where necessary. Statistical significance was considered at P < 0.05 and tendencies are reported when 0.05 ≤ P ≤ 0.1. Values are presented as least square (LS) means ± standard error, unless stated otherwise.

A generalized linear mixed model (PROC GLIMMIX) was used to assess the effect of supplementation (Lacto, Placebo) under stressed (S) versus non-stressed (NS) conditions. For each outcome, body weight and baseline values (i.e., behavior or physiological measures collected at 18 woa) were used as covariates. Differences between LS means were compared pairwise using a Tukey–Kramer adjustment. Scatter plots of studentized residuals against predicted values, and treatment values and a Shapiro–Wilk test of normality were used to confirm the assumptions of the variance analysis. To detect possible outliers, studentized residuals outside a ± 3.4 envelope were used. To identify whether blood measurements were interrelated with the severe FP phenotype, an additional GLIMMIX model was performed for each blood parameter with the phenotype as a fixed effect.

**Data availability**

All data necessary to evaluate the conclusions of this paper are available from the following online repositories: https://dataverse.scholarsportal.info/privateurl.xhtml?token=9050f262-d6bc-4bcd-8699-9121976dddc8.

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Author contributions
C.M., N.v.S., W.K., P.F. and A.H.-M. conceived and designed the experiment. W.K., P.F. and A.H.-M. secured the funding for the experiment. C.M. and N.v.S. performed the experiment and collected the data at the research station. A.B., D.F., J.M.G. and M.F.M. processed the samples. C.M. and A.B. conducted the statistical analysis. C.M. wrote the original draft. C.M., N.v.S., A.B., D.F., J.M.G., J.B.K., W.K., M.F.M., A.K.S., P.F. and A.H.-M. reviewed and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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