The inflammatory immunity and gut microbiota are associated with fear response differences in laying hens

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ABSTRACT The fear response is a crucial adaptive mechanism for coping with environmental changes, and the individuals have different levels of fearfulness. The purpose of this study was to determine the status of the immune response and gut health in hens with different fear responses. A total of 80 healthy 75-wk-old native Lindian chickens were individually housed in conventional cages and categorized into high (**TH**) and low (**TL**) levels of fearfulness using the tonic immobility (**TI**) test. The immunological status and intestinal health of the laying hens were assessed, and the intestinal microbial community was sequenced using 16S rRNA testing. The results showed that the immune-related genes of interleukin (**IL**)-1β, IL-4, IL-6, and IgG were significantly upregulated in the spleen of TH hens compared with hens in the TL group ($P < 0.01$). The inflammatory immune-related genes Toll-like receptor (**TLR**)2, TLR4, nuclear factor (**NF**)-κB, inducible nitric oxide synthase (**iNOS**), cyclooxygenase (**COX**)-2, IL-10, and IgG were significantly increased in the intestinal tract, whereas IL-4, IgA, and the intestinal barrier gene claudin-4 were significantly decreased in TH hens ($P < 0.05$). In addition, serum concentrations of IL-1β, IL-6, IL-10, interferon (**IFN**)-α and IgG were significantly higher in TH hens ($P < 0.01$). A high fear response also led to changes in gut microbial diversity, with a higher Simpson's index and lower β-diversity similarity than hens with a low-fear response ($P < 0.05$). The TH group showed an increase in 8 genera, including *Bacillaceae* and *Coprococcus*, whereas the genus *Anaerorhabdus* decreased ($P < 0.05$). The gut microbiota has also been associated with gut barrier genes, and inflammatory cytokines. *Bartonella* stimulates IL-1β and IgG secretion, whereas *Lactobacillus* inhibits IL-6 secretion, and *Coprococcus* and *Subdoligranulum* are associated with the maintenance of intestinal barrier function. The results of this study suggest that laying hens with high fear response levels have a more sensitive immune response and a more enriched gut microbiota, which may have positive effects on adapting to a complex environment.

Key words: fearfulness, tonic immobility, inflammatory immunity, gut microbiota, laying hen

INTRODUCTION

Fearfulness is an important behavioral and physiological response, which can be defined as an individual's predisposition to be easily frightened (Jones, 1996). This trait helps animals cope with threats and complex environments. However, disproportionate fear responses negatively impact animal welfare and health (Jones, 1996; Dumontier et al., 2022). Poultry may panic or even die in response to stimuli such as shadows cast by airplanes or sudden loud noises (Grandin, 1989; Jones and Waddington, 1992; Purcell et al., 1988). In commercial farming systems, fearfulness reduces egg production and increases the sensitivity of laying hens to environmental changes (Weary et al., 2017). A strong fear response can also increase the incidence of harmful behaviors such as feather pecking (De Haas et al., 2013; Grams et al., 2015) and aggression due to overcrowding (Gray et al., 2020). Harsh handling by handlers was shown to induce intense anxiety responses in broilers (Casey-Trott et al., 2015; Wei et al., 2020). Overcrowded and confined living conditions reduce the adaptability of poultry, and the excessive suppression of fear responses compromises animal welfare (Dwyer, 2004). As a potential stressor, excessive fear can disrupt the immune balance in animals and increase the risk of infection and metabolic disorders (Glaser and Kiecolt-

Glaser, 2005; Dhabhar, 2014). The fear response typically manifests as active defense (including fighting or active avoidance) or passive defense (including freezing response and tonic immobility) (Coleman, 2012). The freezing response manifests as momentary body stiffness and is accompanied by increased energy metabolism and an active endocrine system (Steen, et al., 1988; Cabanac and Aizawa, 2000).

Tonic immobility (**TI**) is an innate, unlearned antipredator strategy that was previously considered to be a state of "fear paralysis" in which the animal becomes immobile due to fear (Holmes, 1929). Many tests have been developed to quantify the fear sensitivity of animals, including open field tests, restraint experiments, novel object tests, and human contact experiments (Forkman, et al., 2007; Franco, et al., 2022). However, the TI test is a simple and effective method of measuring fearfulness in chickens. This method is based on a feigned death strategy of chickens to avoid predators for chickens. The TI test induces tonic immobility through standardized measures and has a high success rate and reproducibility. The duration of tonic immobility is positively correlated with the level of fearfulness (Jones, 1996). Consequently, the TI test is often used as a simple and practical field method to effectively quantify fear levels in poultry (Crawford, 1977; Hazard, et al., 2008).

The gut serves as the primary site for digestion and nutrient absorption. However, it also harbors a complex microbiota that helps to regulate the body's immune responses. Extensive research suggests that changes in the gut microbiota are associated with emotional states, as seen in depression, anxiety, and fearfulness disorders, where the gut microbiota composition differs from that of healthy individuals (Dinan and Cryan, 2013). Voluntary wheel running exercise was shown to alleviate depressive and anxious behaviors in mice by positively affecting the gut microbiota and reducing levels of intestinal pro-inflammatory cytokines (Williams, et al., 2023). Changes in the gut microbiota structure are thought to influence intestinal immune responses (Zhou et al., 2020). The symbiotic relationship between the gut microbiota and the host plays a crucial role in which the microbial composition and structure help regulate the host's immune system, while the host's intestinal immunoreaction contributes to improving the composition and diversity of the gut microbiota. However, whether variation in fear responses in domestic chickens are related to the body's immune responses and gut microbiota remains unclear. Therefore, this study aimed to elucidate the relationship between fear behavior with immune responses and gut microbiota by comparing native laying hens exhibiting high and low degrees of fearfulness. We predicted that hens with a high level of fear that did not influence production performance could exhibit sensitive immunological and inflammatory responses. Additionally, they might possess a complex intestinal microbiota to help maintain the homeostatic balance for sustaining body health and adapting the housing environment.

MATERIALS AND METHODS

Ethics Statement

The experiment was conducted with the approval of the Animal Care and Use Committee of Northeast Agricultural University (NEAU-[2011]-9) and with the project number (NEAUEC20220251). The animal husbandry and slaughter procedures were in accordance with the requirements of the Chinese Ministry of Science and Technology and the "Guide for the Care and Use of Laboratory Animals (2006) No. 398″ published by the National Academies Press.

Study Animals and Management

For this experiment, 80 native Lindian chickens (Harbin Breeding Farms, China) at 75 wk of age were selected as the experimental model. The main production area of Lindian chicken is in Lindian County, Heilongjiang Province, China (Zhang, et al., 2015; Wang, et al., 2017). Lindian chickens are a local dual-purpose breed in the northern cold region and breed for both meat and eggs. They have advantages such as cold resistance, robust vitality and tolerance to coarse feed. The chicken has a medium sized body and relatively slender legs and some may have leg feathers. The skin is white and the feathers are generally thick, with dominant colors of deep yellow, pale yellow and black. Males often have a golden yellow color with long tail feathers that are black (Zhang et al., 2015; Wang et al., 2017). Each hen was fitted with a digital leg band on the left leg for easy identification and tracking, and the experiment was conducted in a controlled environment in enclosed hen houses. Each hen was individually housed in conventional cages measuring 48 × 42 × 35 cm (length × width × height). A consistent light cycle of 14 h per day (from 5:00 am to 19:00 pm) was maintained, with light intensity ranging from 15 to 21 lux. The ambient temperature in the henhouse was maintained between 18 and 21°C, and the relative humidity was kept within the range of 50 to 70%. Commercial diets for laying hens were provided, and each cage was equipped with a nipple drinker to ensure free access to feed and water throughout the experiment.

Tonic Immobility Test

At 76 wk of age, the laying hens (n = 80) underwent TI testing according to the experimental protocol described by Salzen (1963). An experimenter carefully held the hen and placed it face up in a U-shaped groove. The chicken's head was covered with one hand while the other hand gently pressed down on the chicken's chest for 15 s. The experimenter waited for the chicken to stop struggling before slowly removing the hand that was pressing on the chest and walking away until he was out of sight of the chicken. The time taken for the chicken to return to normal behavior from the tonic immobility state was recorded. If the time was less than 15 s, the

induction was repeated. Induction that had to be repeated more than 3 time were considered a failure. The maximum duration of the experiment was 15 min. Based on the TI duration results, the laying hens were categorized into high-fear (**TH**) and low-fear (**TL**) groups, each consisting of 9 chickens.

Sample Collection

After 3 d of behavioral testing, blood was drawn from a total of 75 laying hens (5 chickens were excluded because the maximum number of induction trials was exceeded in TI test) using the wing vein blood collection method, and serum was subsequently separated from the collected blood samples. Subsequently, the target 18 chickens were euthanized through cervical dislocation. The spleen, small intestine segments, and cecal contents were dissected from each bird. These tissue samples were rapidly frozen using liquid nitrogen and stored at -80°C for further experiments.

Measurement of Serum Immunological Marker Levels in Chickens

The serum concentrations of immunological markers interleukin (**IL**)-1β, IL-4, IL-6, IL-10, interferon (**IFN**)-α, IFN-γ, tumor necrosis factor (**TNF**)-α and IgG in all laying hens (n = 75) were quantified using ELISA kits according to the manufacturer's instructions (Shanghai Xinle Biotechnology Co., Ltd, Shanghai, China). Briefly, the standard curve was constructed using different concentrations of standard solutions. A 10 μL of serum sample was added to microtiter wells, and optical density was measured at 450 nm using a SpectraMax Plus 384 Enzyme Marker (Molecular Devices, CA). Serum cytokine concentration in the samples were determined by comparison to the standard curve.

Extraction, Reverse Transcription, and Real-Time Fluorescent Quantitative PCR of Total RNA from Spleen and Intestinal Segments

The total RNA was extracted from spleen and intestinal tissue samples of chickens (n = 9) from TH and TL groups of according to the operating procedures described in the RNAiso Plus kit manual (Takara, Dalian, China). The extracted total RNA was then resuspended by 50 μL of 0.1% DEPC-treated water. The spectrophotometer (Gene Quant 1300/100, Biochrom Ltd., Cambridge, UK) was used to determine the concentration of total RNA at OD260 and the purity of the total RNA by measuring the OD260/OD280 ratio. For cDNA synthesis, the FSQ-101 Reverse Transcription Kit (TOYOBO, Osaka, Japan) were used, and then the harvest cDNA was stored at -20°C for future use.

The National Center for Biotechnology Information (**NCBI**) site was used to design primers for genes involved in barriers, such as zonula occludens protein-1

(**ZO-1**), zonula occludens protein-2 (**ZO-2**), claudin-1, claudin-3, claudin-4, occludin, E-cadherin, and mucin-2, inflammation-related factors including nuclear transcription factor-kappa B (**NF-κB**), inducible nitric oxide synthase (**iNOS**), cyclooxygenase-2 (**COX-2**), Toll-like receptor 2 (**TLR2**), Toll-like receptor 4 (**TLR4**), and inflammatory cytokines including interleukin-1β (**IL-1β**), interleukin-4 (**IL-4**), interleukin-6 (**IL-6**), interleukin-10 (**IL-10**), interferon-alpha (**IFN-α**), interferon-gamma (**IFN-γ**), tumor necrosis factor-alpha (**TNF-α**), immunoglobulin A (**IgA**) and immunoglobulin G (**IgG**) (STable 1). The qRT-PCR reactions were performed on the Light Cycler 480 qPCR system (Roche, Rotkreuz, Switzerland) using β-actin (NM_205518.2) as an internal reference gene, and each 10 μL reaction mixture contained 5 μL 2X NovoStrart SYBR qPCR SuperMix Plus (Novoprotein, Suzhou, China), 1 μL diluted cDNA, 0.3 μL of each primer (10 μM), and 3.4 μL RNase-free water. The qPCR conditions were as follows: initial heating to 95°C for 1 min, followed by 40 cycles of 95°C for 20 seconds and 60°C for 1 min. Relative mRNA expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method.

Extraction and Library Construction of Gut Microbiota 16S rDNA, and Sequencing Analysis

The total genomic DNA samples of chickens from TI and TH groups (each group selected 6 chicken samples from 9 chickens) were extracted using the OMEGA Soil DNA Extraction Kit (M5636-02) (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions and stored at -20°C prior to further analysis. The quantity and quality of the extracted DNA were measured using the NanoDrop NC2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and quality assessment was performed using agarose gel electrophoresis. The V3-V4 region of the bacterial 16S rRNA gene was amplified by PCR using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGAC-TACHVGGGTWTCTAAT-3′). To enable multiplex sequencing, sample-specific 7-bp barcodes were incorporated into the primers. The PCR reaction mixture included 5 μL of buffer (5×), 0.25 μL of Fast pfu DNA polymerase (5U/μL), 2 μL of dNTPs (2.5 mM), 1 μL of each forward and reverse primer (10 μM), 1 μL of DNA template, and 14.75 μL of ddH2O. The thermal cycling program consisted of an initial denaturation at 98°C for 5 min, followed by 25 cycles of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 min. The PCR products were purified using Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA). After individual quantification, the amplicons were pooled in equimolar amounts and subjected to paired-end sequencing (2 × 250 bp) using

the Illumina NovaSeq platform and NovaSeq 6,000 SP Reagent Kit (500 cycles). Following LC-Bio's instructions, the samples were sequenced on the Illumina NovaSeq platform. Paired-end sequences were assigned to samples based on their unique barcodes, and the paired-end sequences were truncated by removing the barcode and primer sequences. FLASH was used for merging paired-end reads. Raw reads were subjected to quality filtering under specific conditions using fqtrim (v0.94) to obtain high-quality clean tags. The chimeric sequences were screened using Vsearch software (v2.3.4). After denoising with DADA2, feature tables and feature sequences were obtained. Based on the SILVA (release 132) classifier, the relative abundance of features in each sample was normalized. Alpha and Beta diversity were calculated using QIIME2, analyzing species diversity and evenness in each sample through Chao1, Observed species, Goods coverage, Shannon, and Simpson metrics. Sequence alignment was conducted using BLAST alignment, and representative sequences were annotated against the SILVA database.

Statistical Analysis

All data were organized and statistically analyzed using Excel 2016 and IBM SPSS 23.0. The normal distribution of data was assessed using the Kolmogorov-Smirnov test. The duration of TI was not normally distributed, so the non-parametric Mann-Whitney U test was used to detect the difference in the duration of TI in laying hens in the high and low fear groups. The serum concentrations and qRT-PCR results of the TH and TL groups were compared using the independent samples t-test. Correlation analysis of serum ELISA results and the TI results of 75 experimental chickens was performed using the Spearman's test. Correlation between gut microbial genus level with serum results, gut barrier PCR results, and gut inflammation gene expression levels were analyzed using Spearman's test. The results were negatively correlated when r value was < -0.2 and positively correlated when r value was > 0.2. The results are expressed as "mean ± SEM". P-values of < 0.05 were considered statistically significant, and P-values of < 0.01 were considered highly statistically significant.

RESULTS

Tonic Immobility Test

Seventy-five experimental chickens successfully exhibited tonic behavior in the TI test, while 5 chickens because the maximum number of induction trials was exceeded (3 trials). The duration of freezing behavior in the 75 Lindian chickens showed distinct characteristics, with a median of 335 s, a maximum of 900 s, and a minimum of 23 s (Figure 1).
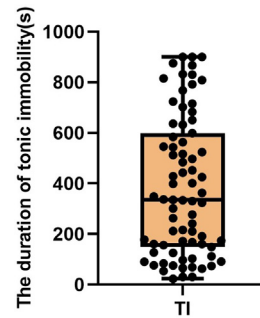

Figure 1. Distribution of the freezing response duration of chickens in tonic immobility (TI) test (n = 75). (Box plot of the duration distribution).

Analysis of the Correlation Between TI duration and Concentrations of Serum Immunological Markers

The results of the correlation analysis between the concentrations of serum cytokines and IgG in laying hens and the duration of the TI test are shown in Figure 2. Among them, the serum concentrations of IL-10, IFN-α, and IFN-γ showed statistically significant positive correlations with the duration of TI in laying hens ($P < 0.05$), whereas no significant correlations were found in other indicators ($P > 0.05$).

Serum Cytokines Levels in Laying Hens With Different Fear Responses

The TI test results indicated that the duration of TI in the TL group (s < 90) was highly significantly shorter (65.56 ± 5.30) than that in the TH group (858.67 ± 12.57) (n = 9) (s > 800) ($P < 0.001$) (Figure 3A). Measurements of serum immune-related markers showed that the levels of IL-1β, IL-6, IL-10, IFN-α, and IgG in the serum of chickens in the TH group were statistically significant higher than those in the TL group ($P < 0.05$) (Figures 3B, 3D, 3E, 3F, 3I). There were no statistically significant differences in other indicators ($P > 0.05$).

Expression of Inflammation-Related Factors in Laying Hens With Different Levels of Fearfulness

The expression levels of Toll-like receptors (**TLRs**) and other immune regulatory factors in chickens were investigated (Figure 4). In the spleen (Figure 4A), no significant differences were observed in the expression of TLRs and pro-inflammatory factors between the TH and TL groups. However, the analysis of inflammatory cytokine gene expression in the spleen (Figure 4B) revealed a statistically significant increase in the relative expression levels of IL-1β, IL-6, IL-4, and IgG in the TH group compared to the TL group ($P < 0.05$). However, there were no significant changes in the relative expression levels of IL-8, IFN-γ, TNF-α, IL-10, and IgA. The TH group exhibited a statistically significant upregulation in the expression of TLR2 in the ileum ($P < 0.05$)
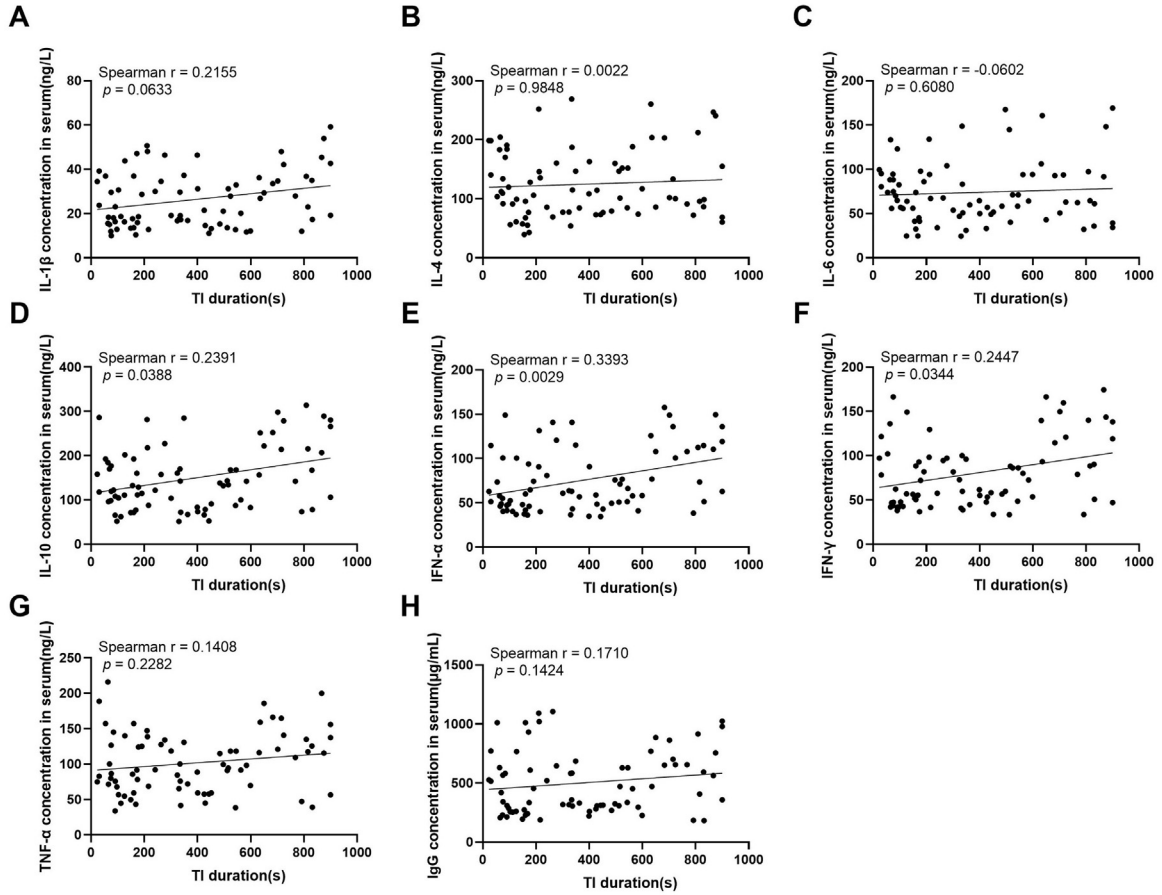
Figure 2. Results of Spearman's correlation analysis between serum cytokines and the duration of tonic immobility (TI) (n = 75). IL-10, IFN-α, and IFN-γ concentrations were significantly positively correlated with the duration of TI in laying hens in the TI test ($P < 0.05$).

and a highly statistically significant upregulation in the expression of TLR4, NF-κB, iNOS, and COX-2 ($P < 0.01$) (Figure 4C). Regarding the ileal inflammatory cytokine gene indices (Figure 4D), the TH group showed

highly statistically significant lower relative expression levels of IL-4 and IgA ($P < 0.01$) compared to the TL group. Conversely, the relative expression levels of IL-10 and IgG were statistically significant higher in the TH
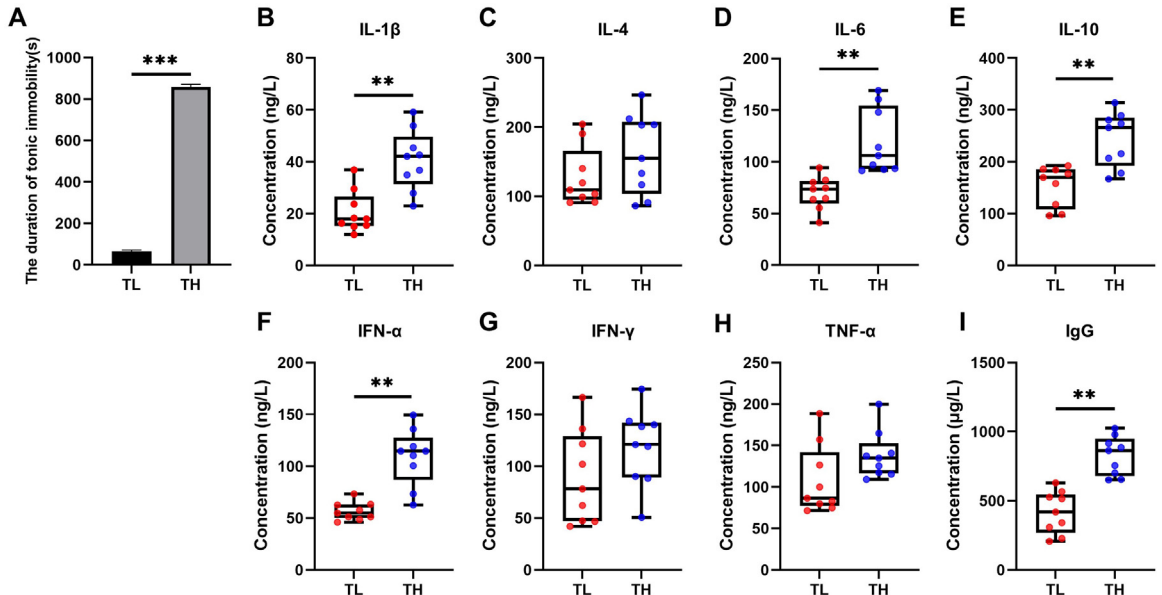

Figure 3. Differences in TI duration and serum concentrations of immunomarkers in high-fear and low-fear hens (Independent t-test) (n = 9). (A) Difference in tonic immobility duration between chickens with different fear levels. (B) Serum concentrations of IL-1β, (C) IL-4, (D) IL-6, (E) IL-10, (F) IFN-α, (G) IFN-γ, (H) TNF-α, and (I) IgG. *$P < 0.05$, ** $P < 0.01$. and ***$P < 0.001$.
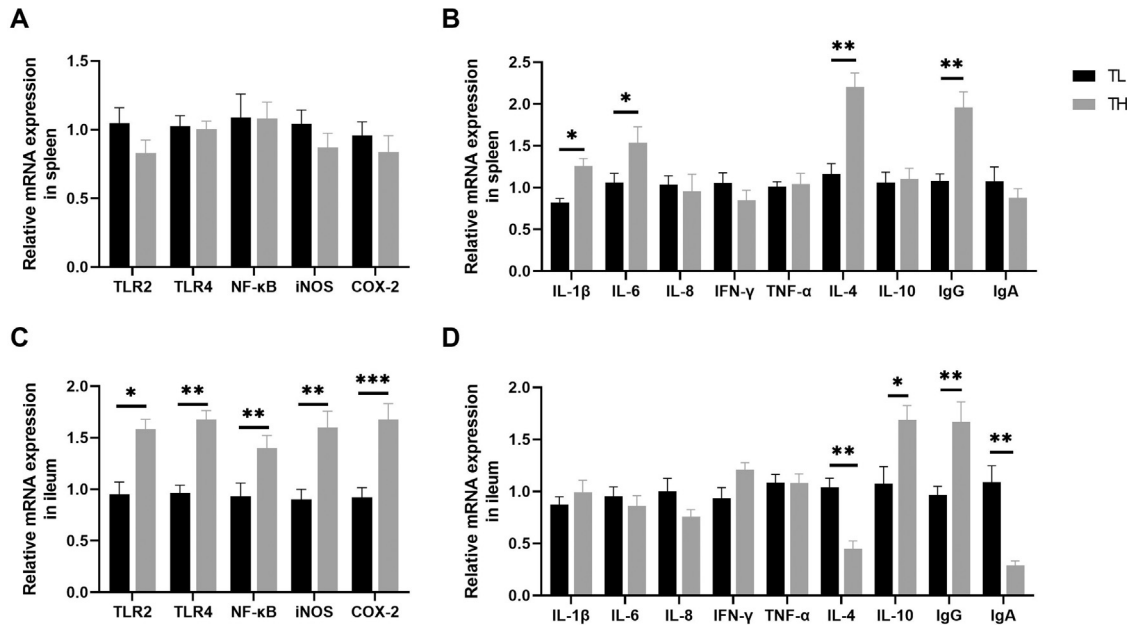
Figure 4. The mRNA Expression levels of inflammatory factor in the spleen and intestinal tissues from chickens with high and low fear hens (Independent t-test) (n = 9). (A) and (B) The relative expression levels of inflammatory and cytokine mRNA in chicken spleens. (C) and (D) The relative expression levels of inflammatory and cytokine mRNAs in the intestinal. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

group than in the TL group ($P < 0.01$). No statistically significant changes were observed in other factors ($P > 0.05$).

Effects of Different Fear Levels on the Expression Levels of Intestinal Barrier Genes

The expression levels of Intestinal barrier-related genes in laying hens from both the low and high fear groups are depicted in Figure 5. Laying hens in the TH group exhibited a significant increase in the expression of mucin2 compared to the TL group ($P < 0.05$). Conversely, claudin-4 expression was significantly reduced in the chickens from the TH group compared to the TL group ($P < 0.05$). However, no statistically significant differences were observed in the expression levels of other proteins, including claudin-1, claudin-3, E-cadherin, ZO-1, and ZO-2 between the 2 groups ($P > 0.05$).
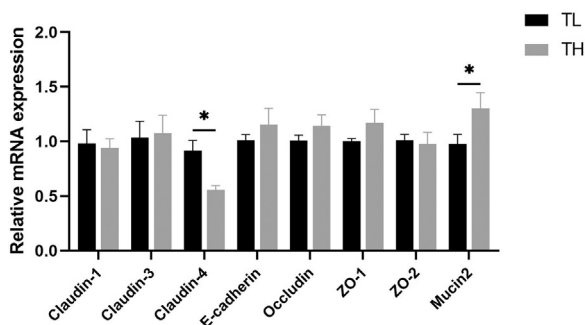

Figure 5. The mRNA expression levels of intestinal barrier factors in chickens in the high-fear and low-fear hens (Independent t-test) (n = 9). *$P < 0.05$, **$P < 0.01$. and *** $P < 0.001$.

Gut Microbiota Analysis of Laying Hens With Different Levels of Fear

As the sequencing reads accumulated, the observed amplicon sequence variants (**ASVs**) curves for all samples reached a state of saturation (Figure 6A), indicating sufficient sequencing coverage for subsequent analyses. Figure 6B illustrates the ASV outcomes of the high fear response and low fear response groups of chickens. A total of 3,293 ASVs were shared between the TL response group and the TH response group, while 8,487 ASVs were exclusive to the former, and 9,423 ASVs were unique to the latter. At the phylum level, the composition was predominantly dominated by *Bacteroidetes, Firmicutes*, and *Proteobacteria*. Notably, the proportion of *Firmicutes* in the TH group (33.89%) was significantly increased compared to the TL group (23.67%). Conversely, *Bacteroidetes* accounted for a reduced proportion (48.46%) in the TH group, whereas a higher proportion was observed in the TL group (62.25%) (Figure 6C). At the genus level, the prevailing genera were *Bacteroides* (22.23%) and *Lactobacillus* (5.45%). The intestinal microbiota in the TH group showed a notably elevated proportion of *Lactobacillus* compared to the TL group (Figure 6D).

Microbial Community Diversities of Laying Hens

The diversity and differences in the cecum microbiota of the TH and TL laying hens were investigated (Figure 7). The alpha diversity analysis revealed that the Simpson's index values were significantly higher in TH hens (ranging from 0.96 to 0.99, median 0.98) than
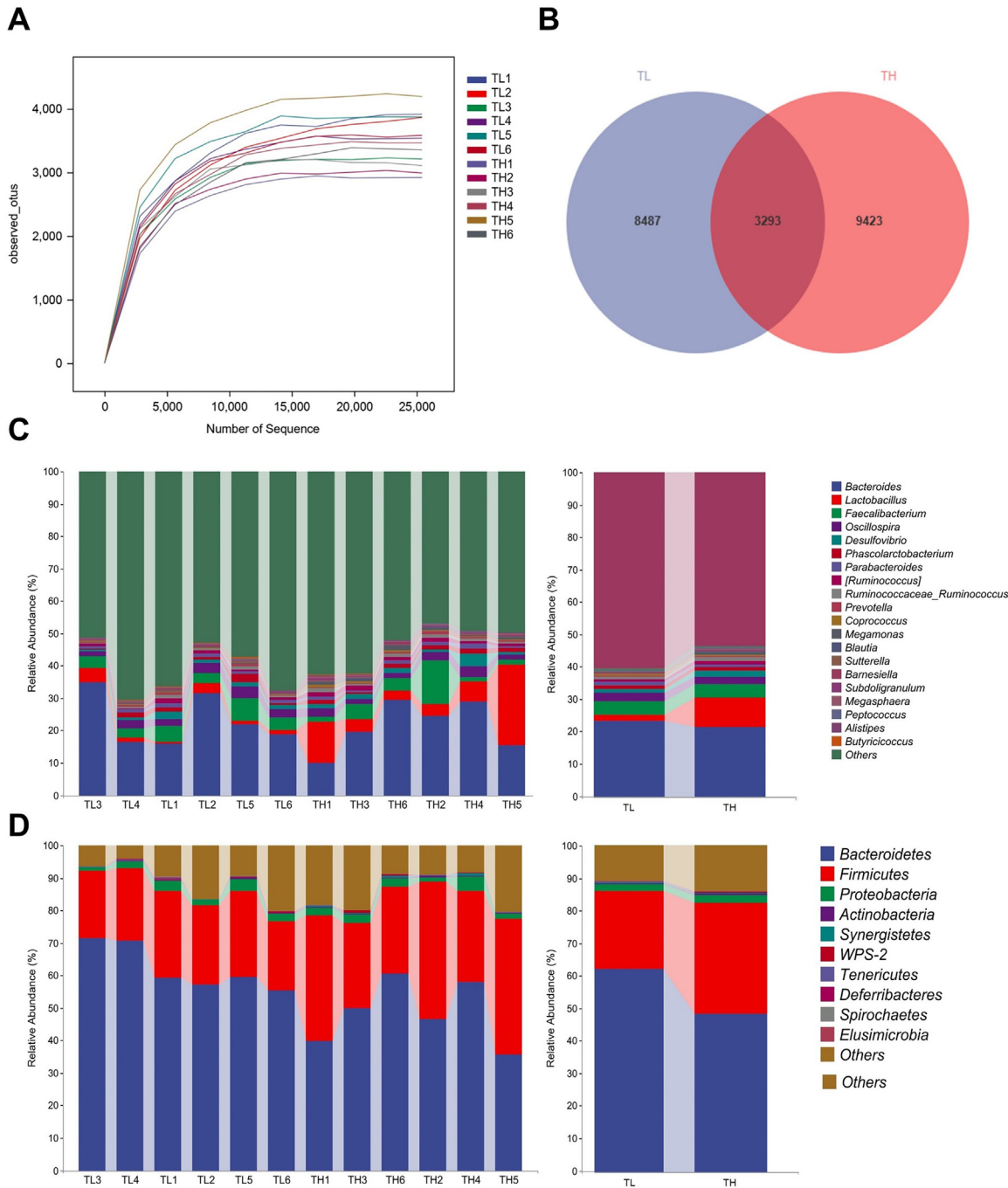
Figure 6. Analysis of intestinal microorganism species composition. (A) Sparse curve illustrating the intestinal flora of hens in both groups. (B) Venn analysis of amplicon sequence variants (**ASVs**) in hens from the TH and TL groups. (C) Phylum level differences in gut microbial components of chickens in both groups. (D) Genus level differences in gut microbial components of chickens in both groups.

in TL hens (ranging from 0.86 to 0.98, median 0.94) ($P <$ 0.05), and there were no significant differences in other indicators (Figure 7A). Differences in gut microbiota between groups were compared using beta diversity analysis. Principal coordinate analysis (**PCoA**) and nonmetric multidimensional scaling (**NMDS**) were used as unconstrained sorting techniques to reduce multidimensional microbial data to lower dimensions (Figures 7B and 7C). The gut microbiota was clearly separated between the high and low fear groups, and showed a clear separation of sample clusters within the same group on the PCoA plot. These results may have

been due to fear, leading to changes in the composition of the gut microbiota, thus affecting gut health.

Linear Discriminant Analysis Effect Size Analysis

The Figure 8 presents the LEfSe analysis results, which revealed significant differences in abundance between the TL and TH groups. Evolutionary branching maps of differential species employs concentric circles radiating from the innermost to outermost layers,
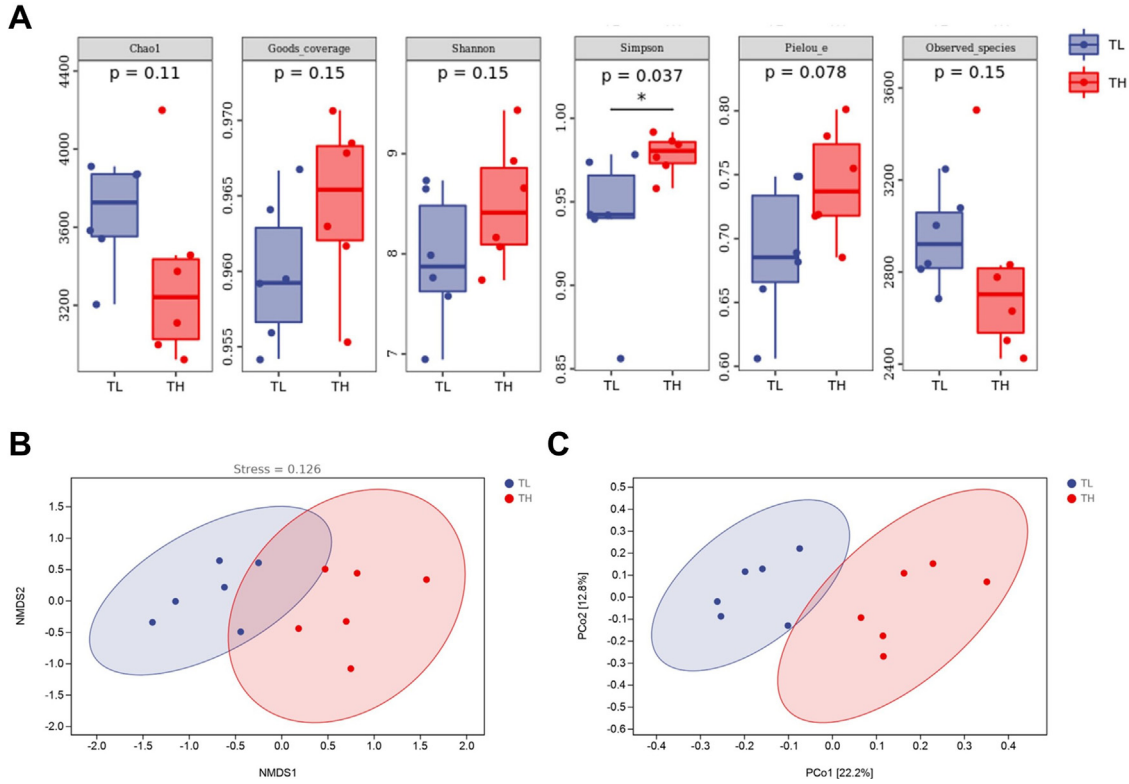
Figure 7. Species Diversity Analysis. (A) Alpha-diversity Analysis. (Independent t-test) (B) Non-metric multidimensional scaling (**NMDS**). (C) Principal coordinates analysis (PCoA).

representing 7 taxonomic levels: domain, phylum, class, order, family, genus, and species. Each node within the diagram corresponds to a specific taxonomic classification at the respective level, with its size proportional to the species' abundance within the samples. Figure 8A shows the differential microbial species found in the intestines of TL and TH chickens through LEfSe analysis. Figure 8B illustrates a bar plot depicting the significant differences in species abundance between the 2 groups. A total of 18 bacterial taxa were substantially different between the intestines of low and high fear response chickens (LDA score > 3.0, $P < 0.05$). Among

them, 17 bacterial taxa, including the *Firmicutes* phylum, *Bacilli* class, *Lactobacillales* and *Turicibacterales* orders, *Bacillales* family, *Lactobacillaceae, Dehalobacterium, Planococcaceae, Turicibacteraceae, Christensenellaceae*, and *Bacillaceae* families, as well as *Lactobacillus, Dehalobacteriaceae, Megamonas, Turicibacter, Megasphaera, Coprococcus*, and *Blautia* genera, were significantly enriched in the intestines of TH hens compared to TL hens (LDA score > 3.0, $P < 0.05$). The remaining bacterial taxon, *Anaerorhabdus* genus, was predominantly enriched in the intestines of low fear response chickens (LDA score > 3.0, $P < 0.05$).
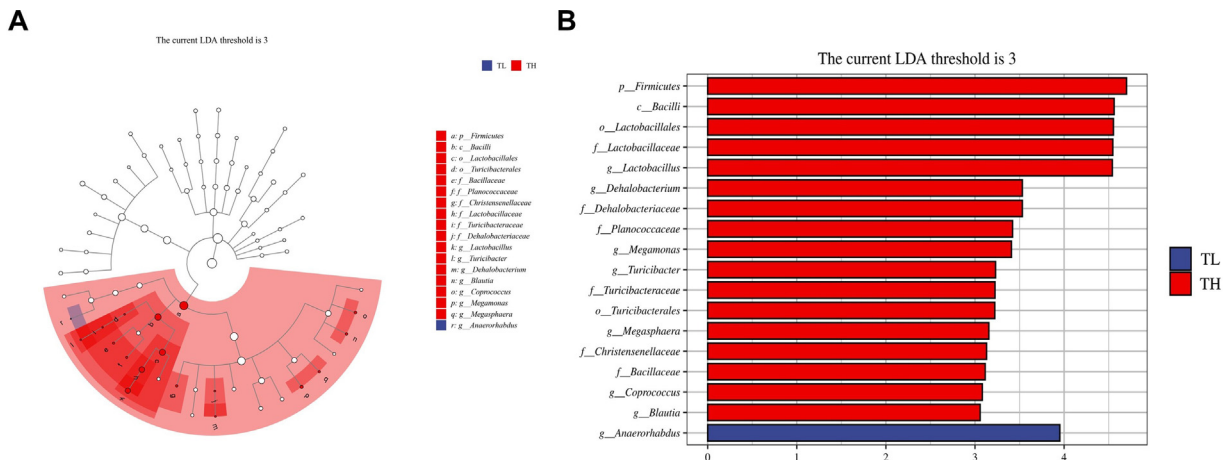

Figure 8. Linear discriminant analysis effect size (**LEfSe**) analysis. The LEfSe analysis chart revealed a significant difference in population abundance between the TH group and the TL group ($P < 0.05$). Specific bacterial groups with LDA scores of > 3 encompassed various taxonomic levels, including phylum, class, order, family, genus, and species.

Correlation Between of Intestinal Microbiota, Immune Indicators and Intestinal Barrier Genes in Laying Hens

Spearman's correlation analysis was performed to investigate the interaction between intestinal bacteria and intestinal and systemic immunity, as shown in the Figure 9. In the serum markers of immune responses to inflammation, *Barnesiella* was significantly positively correlated with serum concentrations of IL-1β and IgG

($P < 0.05$, $r > 0.2$), *Lactobacillus* was significantly negatively correlated with IL-6 ($P < 0.05$, $r < -0.2$), and *Megamonas, Megasphaera* and *Blautia* showed a significant negative correlation with IL-4 and IL-6 serum levels ($P < 0.05$, $r < -0.2$) (Figure 9A). Regarding to the intestinal barrier genes, *Coprococcus* and *Subdoligranulum* showed a significant positive correlation with mucin2 ($P < 0.05$, $r > 0.2$), while *Oscillospira* had a significant negative correlation with mucin2 ($P < 0.05$, $r < -0.2$) and *Megamonas* had a significant negative correlation with
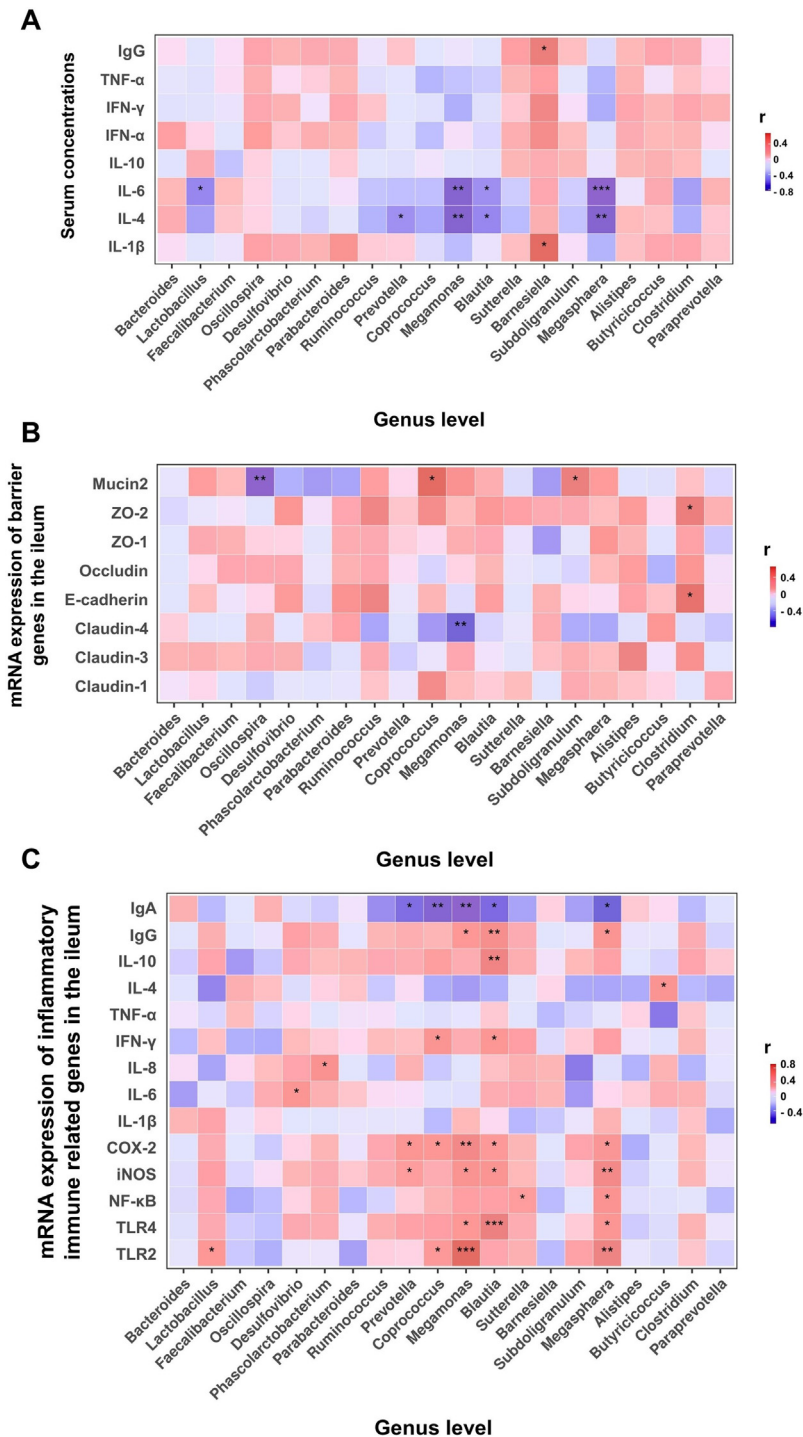

Figure 9. Spearman correlation analysis of serum, tissue inflammation and cytokines and intestinal microbial genus level in laying hens. (A) Correlation analysis of serum immune factor content and genus level. (B) Correlation analysis of gut barrier genes and genus level. (C) Correlation analysis of gut inflammation, cytokines, other genes, and genus level. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

claudin-4 ($P < 0.05$, $r < -0.2$). Clostridium was significantly positively correlated with E-cadherin and ZO-2 ($P < 0.05$, $r > 0.2$) (Figure 9B). At the level of genes of inflammatory immunity genes, *Megamonas* had a significant positive correlation with TLR2, TLR4, iNOS, COX-2 and IgG ($P < 0.05$, $r > 0.2$), and *Blautia* had a significant positive correlation with TLR4, iNOS, COX-2, IFN-γ, IL-10 and IgG ($P < 0.05$, $r > 0.2$), and *Megasphaera* was positively correlated with TLR2, TLR4, NF-κB, iNOS, COX-2 and IgG ($P < 0.05$, $r > 0.2$). A significant negative correlation between IgA and *Prevotella, Coprococcus, Megamonas* and *Blautia* was observed ($P < 0.05$, $r < -0.2$) (Figure 9C).

DISCUSSION

Fear is an instinctive response and emotional experience of animals facing danger and is essential for biological conservation (Beckers et al., 2023). Chickens exhibit personality diversity during domestication, resulting in individuals with different sensitivities to fearful stimuli. This diversity may be influenced by genetics, environmental adaptation and selective breeding, leading to significant differences in fear responses within the same population. These viewpoints are further supported by experimental results showing that chickens of the same species exhibited different fear states in the TI test. The analyzing of the correlation between peripheral blood cytokine levels and fear levels in chickens found that IL-10, IFN-α, and IFN-γ were positively correlated with the levels of fear responses. Thus, these metrics have the potential to be used as biomarkers for evaluating the relationships between fear response and levels of inflammatory immunity in laying hens.

According to a 1996 study by Jones, the fear response helps animals to adapt to their environment. However, intense or chronic fearfulness can cause an immune imbalance (Jones, 1996). The current study found that chickens in the TH group had increased expression of inflammatory factors, including inflammation-related cytokines such as IL-1β and IL-6, compared to the chickens with low fear responses. Increase in serum concentrations of IL-1β and IL-6 may lead to an enhanced inflammatory response that can damage nerves and body tissue structures. IL-1β is an important inflammatory mediator that increases the production of other inflammatory cytokines, such as TNF-α and IL-6, further amplifying the inflammatory response (Weber et al., 2010; Ng et al., 2018). Elevated levels of IL-6 can promote the synthesis and release of acute-phase proteins, which are involved in the adaptation and recovery processes of the body's stress response and play an important role in the regulation of the stress response and immune function (Scheller et al., 2011). Therefore, high fear levels in chickens are associated with increased expression of pro-inflammatory cytokines.

The gut, as an organ connected to the external environment, plays a critical role in nutrient absorption and metabolism, and immune regulation. Studies have shown that emotions can influence gut microbiota and cognitive function through gut-brain axis interactions (Al Omran and Aziz, 2014). Gut-associated lymphoid tissue plays a critical role in intestinal immunity involving antigen-presenting cells, B cells, T cells, and intestinal epithelial cells (Spahn and Kucharzik, 2004). TLR2 and TLR4 are important recognition receptors for various pathogen-associated molecular patterns (**PAMPs**). When the gut is infected, damaged, or has increased permeability, bacteria or their metabolites bind to TLR2 and TLR4 and activate the downstream transcription factor (**NF-κB**) to regulate the inflammatory response. NF-κB promotes the transcription and expression of pro-inflammatory mediators, such as COX-2 and iNOS and inflammation-related cytokines, such as IL-1β, IL-6, TNF-α. The outcomes of inflammation in the gut are highly variable, and we speculate that fear responses are related to gut immunity and gut health. In this study, chickens in the TH group showed significantly increased expressions of TLR2, TLR4, NF-κB, COX-2, iNOS, IL-1β, and IL-10 in the gut, and decreased expression of IL-4 compared to the TL group. This suggests that the inflammatory responses were activated with fearful chickens, perhaps contributing to pathogen clearance, enhanced antimicrobial defense, and wound repair in the gut.

IgA in the gut can bind to and eliminate harmful bacteria, helping beneficial bacteria to colonize the gut (Suzuki et al., 2007). The level of IgA in the gut is closely related to gut health because it acts as a chemical barrier in the gut, isolating harmful organisms (Suzuki et al., 2007). Decreased levels of IgA in the intestine indicate a weakened intestinal immune system, with mucosal damage facilitating the passage of harmful substances through the intestine, causing bodily harm. The mechanical barrier in the intestine consists of intestinal epithelial cells and their tight junctions. The claudin family is a class of cell surface protein molecules that plays an important role in the barrier, of which claudin-4 is located on the cell membrane where it forms channels and tight bands, regulates tight junctions between cells, and plays a critical role in various biological processes such as tumor growth and metastasis, infection, and inflammation (Neesse et al., 2012). Our experimental results showed that the transcript level of claudin-4 was downregulated in more fearful chickens, which might increase intestinal permeability. Some bacterial metabolites could enter the internal environment via the gut and act as antigens to stimulate intestinal lymphoid tissue and enhance the immune response. Studies have shown that exposure of the intestine to toxic substances can lead to the decreased expression of claudin-4, thereby disrupting the intestinal barrier (Pinton et al., 2010). The diverse microbial communities in the gut form a complex microbiota that is critical for host immune homeostasis and provides a competitive barrier against bacterial and fungal pathogens (Clarke et al., 2014). The alpha diversity analysis showed that the Simpson index was significantly increased in the TH

group, indicating that microbial species occupied a greater proportion or dominant position in the gut of high fear chickens, affecting the structure and function of the microbial community in the gut. The beta analysis results showed that the within-group similarity of the microbial community was high between the high and low fear groups, but the population similarity between the groups was low. Therefore, we speculate that individual differences have a significant impact on the diversity of the gut microbiota. The increased abundance of gut microbiota in high fear chickens may disrupt the gut microbiota balance, with increase in harmful bacteria potentially activating gut inflammation.

The microbiota has been increasingly recognized for its ability to influence neurodevelopment, nervous system function, and a variety of complex host behaviors (Al Omran and Aziz, 2014; Vuong et al., 2017). The regulatory role of the gut microbiota can be attributed to its influence on behavior through the microbiota-gut-brain axis. LEfSe analysis was performed to investigate the impact of fear on the gut microbiota structure of chickens. In contrast to previous studies (Huang et al., 2018), we found that fearful chickens exhibited a significant increase in the abundance of Firmicutes at the phylum level. At the genus level, the relative abundance of beneficial bacteria such as *Lactobacillus, Dehalobacteriaceae, Turicibacter, Megasphaera, Coprococcus*, and *Blautia* were significantly increase in TH chickens. *Lactobacillus* helps to promote intestinal health, development, and immune function against harmful microorganisms in the gut. Probiotic studies have shown that the addition of *Lactobacillus* strains helps chicks resist harmful microbial infections and enhances immune competence (Chen et al., 2012; Puetz et al., 2021). Ccolonization with *Lactobacillus* helps to reduce immune stimulation by harmful microorganisms and inhibits IL-4 secretion, suggesting that perhaps fear responses aid in adapting to the environment, including changes in the gut microbiota structure. The increased relative abundance of *Lactobacillus* in TH group chickens may help reduce damage to the gut caused by proinflammatory factors and harmful bacteria. *Megamonas* bacteria can ferment carbohydrates to produce acetate, propionate and lactate. Previous studies reported a high presence of *Megamonas* in the intestines of patients with depression and post-stroke depression (Huang et al., 2021). *Dehalobacterium* is a genus of anaerobic gram-negative bacteria that metabolizes and converts halogenated compounds to produce acetate (Trueba-Santiso et al., 2017). This may indicate that the gut microbiota of the highly fearful hens in this experiment might produce substances that damage the gut and cause inflammation, thus allowing Dehalobacterium to proliferate in the gut and aid in catabolism. In contrast, beneficial bacteria such as *Turisibacter, Coprococcus*, and *Blautia* produce acetate from hydrogen and carbon dioxide. However, although anaerobic gram-negative bacteria of the genus *Anaerorhabdus* are significantly more abundant in the intestinal tract of fearful chickens, few studies have investigated their effects. We suspect that *Anaerorhabdus* may play a driving role in the development of fear behavior in laying hens.

Spearman's correlation analysis showed a positive correlation between *Barnesiella* and serum concentrations of IL-1β and IgG, suggesting that *Barnesiella* may play a role in promoting inflammatory responses and immune reactions, consistent with the findings of Song et al. (2023). Conversely, *Lactobacillus* showed a significant negative correlation with IL-6, indicating its potential as a probiotic to inhibit inflammatory responses. This finding aligns with previous research, as *Lactobacillus* is widely recognized as a probiotic that maintains gut health and reduces inflammation (Wells, 2011). Regarding gut barrier genes, *Coprococcus* and *Subdoligranulum* exhibited a significant positive correlation with mucin2 gene expression, suggesting that these 2 gut bacteria may contribute to maintaining intestinal barrier integrity. Conversely, *Oscillospira* showed a negative correlation with mucin2, implying a potentially adverse effect on intestinal barrier function. The negative correlation between *Megamonas* and claudin-4 gene expression further suggests a negative impact of *Megamonas* on intestinal barrier stability. These findings provide new insight into how the gut microbiota affects intestinal barrier function. At the level of inflammatory immune genes, *Megamonas, Blautia*, and *Megasphaera* showed significant positive correlations with various detected inflammatory immune genes, highlighting their roles in promoting inflammatory responses (Bai et al., 2022; Huang et al., 2022). These bacteria may trigger inflammatory cascades by activating Toll-like receptors (such as TLR2 and TLR4) and inducing the expression of inducible iNOS and COX-2. Additionally, their positive correlation with IgG suggests their involvement in regulating immune responses. Of note, IgA gene expression exhibited significant negative correlations with *Prevotella, Coprococcus, Megamonas*, and *Blautia*. IgA is an important component of intestinal mucosal immunity, and decreased levels might indicate weakened intestinal mucosal immune function. Thus, these gut bacteria may be associated with a decline in intestinal mucosal immune function, potentially increasing the risk of intestinal infections and other gastrointestinal diseases. Complex interactions exist between gut bacteria and the intestinal and systemic immune systems. Different gut bacteria may affect intestinal barrier function, inflammatory responses, and immune responses through various mechanisms. These findings provide important clues for further understanding the role of the gut microbiota in animal health and lay a theoretical foundation for developing targeted strategies for gut microbiota modulation.

In conclusion, the correlation analysis conducted in this experiment sheds light on the relationship between serum immunity and tonic immobility duration in laying hens. The significant positive correlations observed between the serum concentrations of IL-10, IFN-α, and IFN-γ and the duration of tonic immobility suggest their potential as valuable indicators of poultry stress response. These findings could aid in evaluating the

environmental conditions and welfare status of laying hens. The identification of microbial species that correlate with pro-inflammatory or anti-inflammatory levels offers potential focal points for fecal testing or as beneficial additives to daily diets, contributing to the monitoring of laying hen health and growth indicators (Lan et al., 2003). By uncovering these potential stress markers and their associations with immune and gut microbiota parameters, this study provides valuable insights into the stress physiology (Loh et al., 2014). Incorporating these findings into avian breeding programs to select poultry breeds with higher adaptability and lower fear responses could potentially enhance both animal welfare and production efficiency.

CONCLUSIONS

The high level of fear response in hens enhances peripheral and intestinal immune responses and alters gut microbial composition, but may compromise gut barrier function. These changes are more conducive to achieving gut health and adapting to environmental changes.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2024.103816.

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