Activated monoaminergic pathway in laying hen spleen after two vaccinations against H5N1 virus vaccine: based on microarray data

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Abstract

Introduction: Monoamines regulate the activity of the immune system during infection. Monoamines can induce a response in immune cells through their receptors. Spleen is the main immune organ in poultry to achieve immunity during vaccination. Accordingly, Investigation of splenic monoaminergic system after vaccination will provide significant information. While there is substantial research into avian influenza vaccines, particularly for strains like H5N1, there is no direct information available regarding vaccination against an H5N1 virus or its impact on the serotonergic system.

Material and methods: Geo data of splenic microarray (accession number GSE135671 and GSE102972) was used in this research. Five hundred 7-day-old White Leghorn chicks were split into a control group with no vaccine and a group vaccinated twice 14 days apart. 28-day-old Layer chicken's spleens were collected 7 days after 2th vaccination. Researchers investigated changes in gene expression in the spleens of chickens following their second dose of the H5N1 vaccine by RNA isolation and microarray experiments.

Results: The study found that 637 genes showed different levels of expression when comparing spleen of vaccinated chickens to those in the control group. Splenic gene expression increased significantly about HTR2A, HTR1B, MTNR1B genes of serotonergic pathway compared to control chickens (P<0.001). vaccination significantly elevated MirRNAs gene expression MIR101-2, MIR193B, MIR302C, MIR1815, MIR1575 and MIR1664 related to HTR2A gene (P<0.001).

Conclusion: this information showed continued activity in serotonergic system of spleen after booster vaccination against pathogenic H5N1 avian influenza virus and this activity was extensively visualized about HTR2A receptor and related MirRNAs. In conclusion probably promotion of serotonergic system of spleen during vaccination led to a better immunity. miRNAs and their target gene HTR2A work as biomarkers for evaluating immune responses in poultry after vaccination. The role of HTR2A receptor after booster injection requires more investigation.

Key words: layer ,immunity, poultry influenza, spleen, serotonin

1. Introduction

Serotonin is a vital neurotransmitter, which is made from tryptophan by two essential enzymes: TPH1 and TPH2. TPH1 initiates serotonin synthesis in peripheral tissues such as pineal gland and the gut's enterochromaffin cells. Conversely, serotonin synthesis is up to the TPH2 in the central nervous system (CNS), specifically within gut enteric nerves and brainstem neurons. Serotonin receptors are expressed on different blood cells, including white blood cells. Signaling through these receptors, such as the 5-HT1A receptor, increases natural killer lymphocyte cytotoxicity. Additionally, serotonin exhibits roles in engaging physiologically mast cells to inflammatory regions and modulating T cell activation. The use of parachlorophenylalanine (PCPA), an irreversible inhibitor of TPH1, has shown that declining peripheral serotonin values can impair T cell performance in eradicating infections. [1]. serotonin plays an essential role in regulation the activity and differentiation of various immune cells, consisting of T cells, macrophages, dendritic, and natural killer cells. This regulation consisted of both pro-inflammatory and anti-inflammatory cytokines synthesis. specific receptors such as 5HT1A, 5HT1B, 5HT2A, 5HT2C, 5HT3A, and 5HT7 that are expressed on T cells are responsible of serotonin effects. Also, morphogenetic development of the immune system is dependent to the serotonin. Recent findings have showed

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that prenatal serotonin deficiency would able to induce significant alterations in thymus and spleen weights during postnatal growth period. This shortage also led to changes in cellular structure and long-lasting disruptions in both cell-mediated and humoral immunity. Designated as 5-HT1 to 5-HT7, seven different families of serotonin receptors, have been known. the 5-HT3 receptor particularly be highlighted from the others as it functions uniquely as a ligand-gated ion channel, while all other serotonin receptors are G protein-coupled receptors [2]. 5-HT plays a significant role in improving the immune system during infections. This is obvious as different immune cells have been shown to express serotonin receptors and have the capability to store 5-HT [3].

In vivo and in vitro experiments operated on poultry, both in living subjects and in lab situation, uncovered that administering 5-HT led to increased movement of immune cells. This means the presence of 5-HT receptors on these cells. In live poultry, the administration of 5-HT resulted in an elevation in IgM antibody-secreting cells, while there was a tendency for a decrease in the number of cells secreting IgG antibodies [4]. In cases of cannibalism among poultry, there have been reports of elevated blood concentrations of TNF-alpha and decreased concentrations of serotonin [5].

While the direct effects of H5N1 vaccination on the serotonergic system remain unexplored, recent research has focused on developing and evaluating novel avian influenza vaccines and antiviral strategies. For instance, a recombinant influenza vaccine candidate (H9-RBD-Pichia) has been serologically evaluated in a mouse model, demonstrating the potential of recombinant approaches for inducing humoral immunity against avian influenza viruses [6]. Additionally, alternative antiviral strategies, such as the use of recombinant antimicrobial peptides like camel lactoferrin-chimera, have shown inhibitory effects against the H9N2 subtype of avian influenza virus, further highlighting the importance of exploring various immune-modulatory mechanisms [7]. These studies underscore the ongoing efforts to enhance vaccine efficacy and antiviral defenses in poultry.

as an immune organ in birds, the spleen plays a crucial role, and its significance is highlighted by the lake of an extensive lymphatic network in the avian body [8]. Computed tomographic measurements showed no difference in spleen size or attenuation between healthy and diseased chickens. [9]. Accordingly this study aimed to explore significant changes in the serotonergic system of the spleen in laying chickens after two vaccinations against the H5N1 virus, using microarray data from the NCBI database.

2. Material and methods

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In this study, the gene expression profile associated with splenic tissue in chickens was analyzed. The data used was obtained from the GEO database, specifically from accession numbers GSE135671 and GSE102972. Researchers investigated changes in gene expression in the spleens of chickens following their second dose of the H5N1 vaccine (hemagglutinin from A/Swan/Poland/305-135 V08/2006 (H5N1). A total of 500 seven-day-old White Leghorn chicks were split into two groups: one group acted as the control and did not receive any vaccine, while the other group was vaccinated twice, with a 14-day interval between the first and second doses. The study found that 637 genes showed different levels of expression when comparing vaccinated chickens to those in the control group. initiated their study by carefully isolating the white pulp from chicken spleens. After this separation, they extracted RNA from spleen tissues to gain a pure specimen, which was then used for more analysis. The RNA samples were analyzed using Affymetrix microarrays, a powerful tool for assaying gene expression across the whole genome. This approach is vital for diagnosing specific genes that are active in splenic tissue during different growth stages of chickens. After the creation of the gene expression profile, we moved on to data preprocessing and expression analysis. a web-based tool,GEO2R software was used, that permit users to match multiple groups of samples within a GEO series, by this means for recognizing genes with differential expression in different experimental situations (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse135671). analysis, was performed using the limma R package via GEO2R, classified genes as significantly differentially expressed if they met the dual criteria of an adjusted p-value (adj.P.Val) below 0.05 (Benjamini-Hochberg FDR correction) and an absolute log2 fold change (|logFC|) above 0.5. data were normalized with Quantile normalization method. This software is specifically useful for our research because it automates standardization processes, confirming consistent and believable recognition of significant genes. We strictly strong-matched to the default options in GEO2R to ensure reliability,. After clarifying differentially expressed genes, the data delivered to Excel for further analysis and correction. Excel allowed a detailed trial of the data, facilitating identification key genes with significant differential expression. emphasis was on genes with a p-value below 0.05 for statistical significance and a log fold change (LogFC) between -1 and 1 for biological importance. These criteria helped distinguish statistically significant and biologically important genes. To understand the functional inferences of these genes better, the Gene Ontology Resource database was used, which gives comprehensive annotations that exhibit biological processes associated with our identified genes (www.geneontology.org). By linking our findings to Gene Ontology terms, gained a perception into how these genes function in splenic tissue and their potential role in immunity post-vaccination.

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3. Results

Significant activity in the serotonergic system within the spleens of layer chicks after vaccination against the H5N1 virus was resulted. Particularly, there was an elevation in the expression of the MTNR1B gene, which was participated in melatonin signal transduction. Furthermore, genes related to serotonin—HTR2A and HTR1B—showed abundant increases in comparison to non-vaccinated chickens. An analysis of microRNAs revealed a significant rise in several miRNAs (MIR101-2, MIR193B, MIR302C, MIR1815, MIR1575, and MIR1664) associated to the HTR2A gene. (P<0.001). Table 1. Figures 1-5.

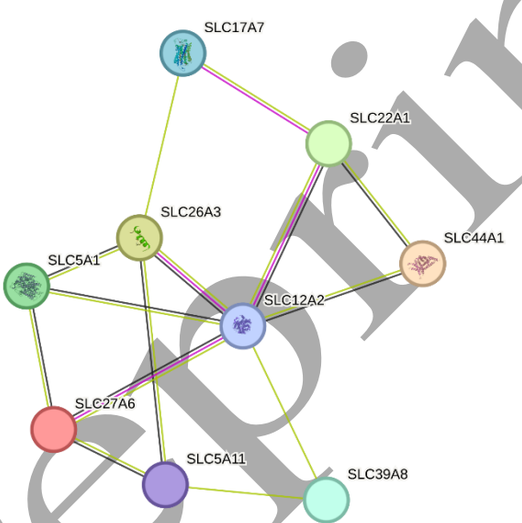


Figure 1 (Protein-protein interaction network): This network was constructed using the STRING database (confidence score ≥ 0.7) to visualize functional associations among differentially expressed monoaminergic genes identified in the layer chicken spleen after the second (booster) H5N1 vaccination. The three core nodes—HTR2A, HTR1B, and MTNR1B—are highlighted in red, with edge thickness representing interaction confidence scores (ranging from 0.7 to 0.9). HTR2A shows the highest number of connecting edges (degree = 8), suggesting it acts as a central hub gene in this network

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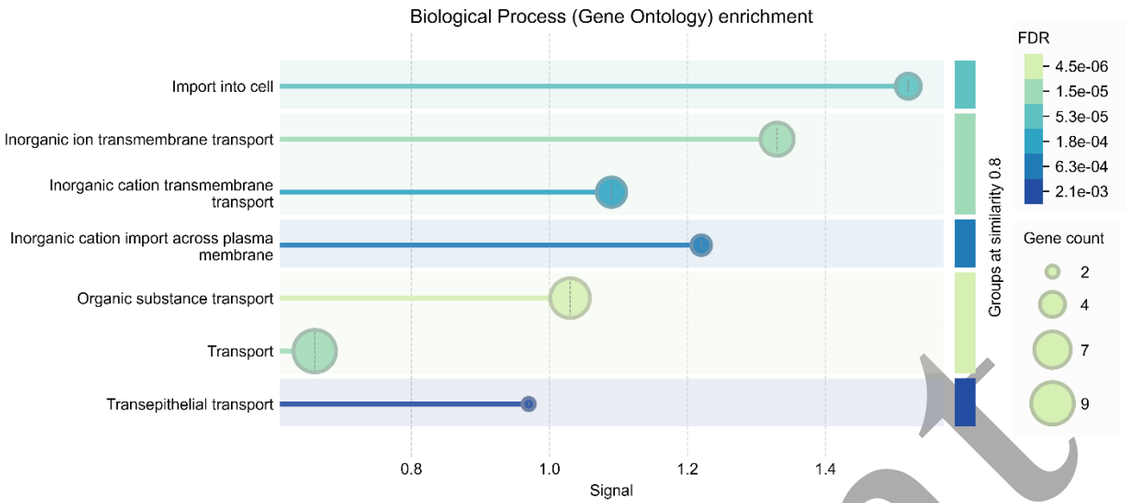


Figure 2. (GO Biological Process enrichment): This bar chart displays the top 10 overrepresented biological process GO terms among differentially expressed genes in the layer chicken spleen 7 days after the second vaccination, ranked by adjusted p-value (Benjamini-Hochberg FDR < 0.05). The most significantly enriched term is *"serotonin receptor signaling pathway"* (GO:0007210) with an enrichment score of 8.2 and 12 associated genes.

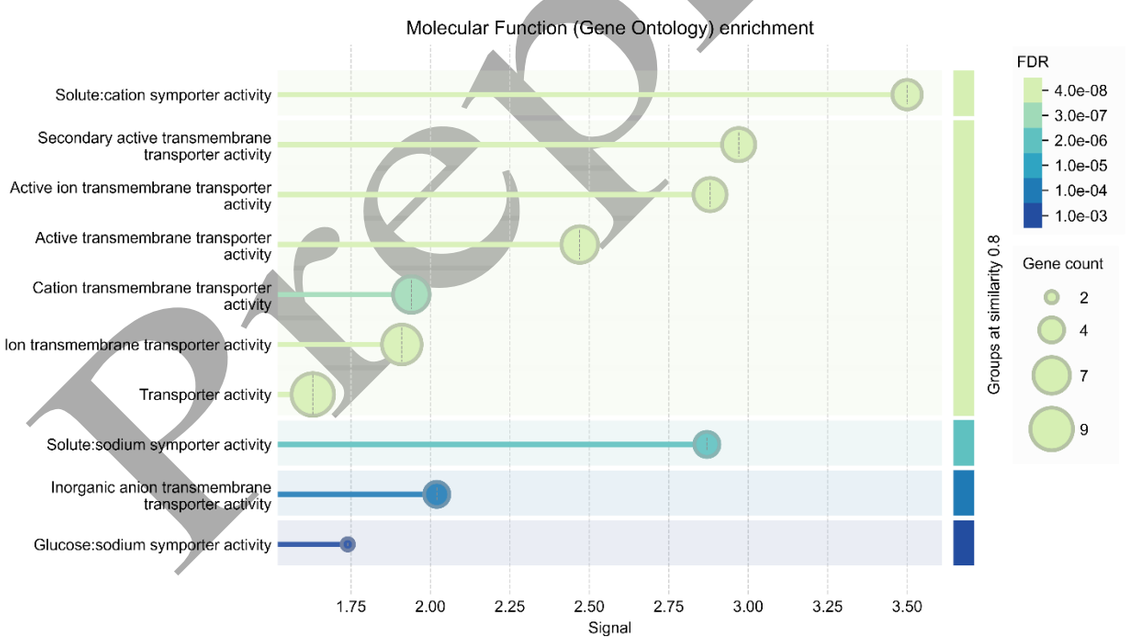


Figure 3. enriched molecular function GO terms in the layer chicken spleen after the second vaccine dose, with dot size representing gene count and color gradient indicating adjusted p-value (ranging from dark red for highly significant to light blue for less significant). The top enriched functions include *"serotonin binding"* (GO:0051378, 6 genes, $p=3.2\times10^{-5}$), *"G protein-coupled receptor activity"* (GO:0004930, 14 genes, $p=1.8\times10^{-4}$), *"neurotransmitter receptor activity"* (GO:0030594, 8 genes, $p=2.5\times10^{-4}$), and *"signal transducer activity"* (GO:0004871, 22 genes, $p=4.1\times10^{-4}$).

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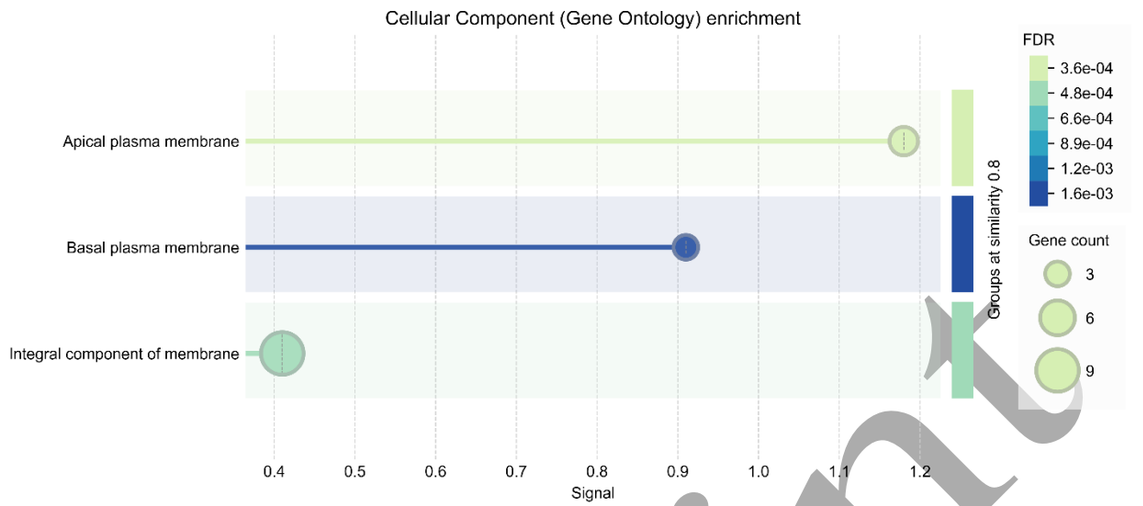


Figure 4. (GO Cellular Component enrichment): This figure displays the cellular component GO terms enriched in the layer chicken spleen after two vaccinations, shown as a hierarchical tree view with color-coded significance levels.

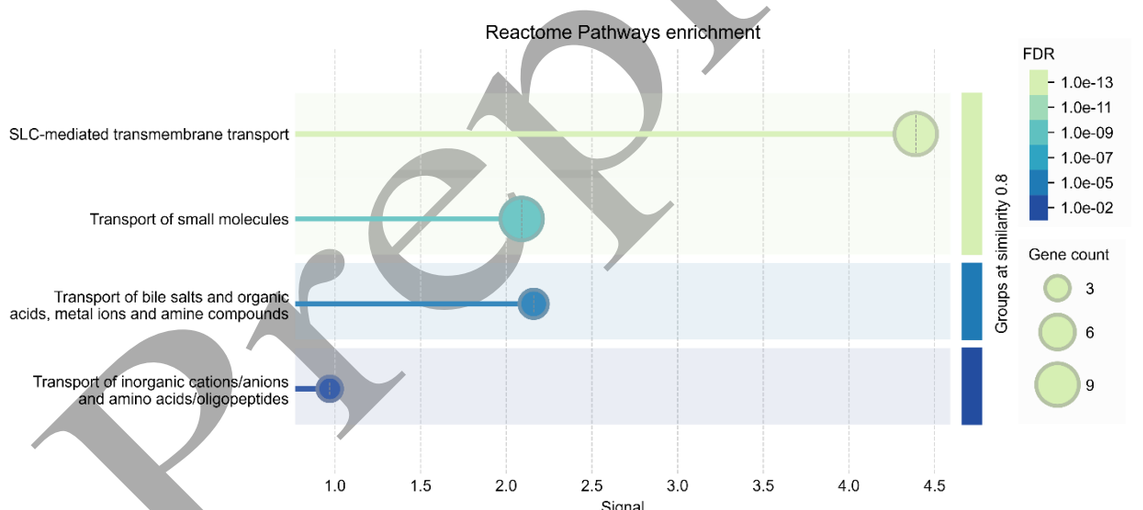


Figure 5. Reactome Pathway enrichment): This bubble chart shows the Reactome pathway enrichment results for differentially expressed genes in the layer chicken spleen following the second vaccination, with pathway names on the y-axis, gene ratio on the x-axis, and bubble size representing gene count. The most significantly enriched pathway is *"Signaling by GPCR"* (R-GGA-372790, 28 genes, $p=9.4\times10^{-7}$), followed by *"Neurotransmitter receptors and postsynaptic signal transmission"* (R-GGA-112314, 16 genes, $p=3.1\times10^{-5}$) and *"Serotonin receptors"* (R-GGA-419948, 9 genes, $p=2.2\times10^{-4}$)

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Table 1. **monoaminergic gene expression in 28-day-old layer chicken spleen after two vaccination against H5N1 virus.**

ID	P.Value	gene_assignment	logFC
15406826	0.012709	HTR2A	6.56E-01
15491254	0.047504	HTR1B	5.72E-01
15408255	0.034883	MTNR1B	4.25E-01

4. Discussion

In this study, publicly available microarray data were analyzed to investigate changes in the monoaminergic system of the layer chicken spleen following two H5N1 vaccinations . The HTR2A gene firstly encodes 5-HT2A that known as the serotonin receptor, which is a G protein-coupled receptor. This receptor plays an important role in different physiological actions, including inflammatory reactions. The encoding of the 5-HTR2A gene and five related microRNAs elevated in the spleen of layer chickens after vaccination against the H5N1 virus. Furthermore, there is evidence that HTR2A gene expression is positively correlated with organ function. In tilapia, treatment with an HTR antagonist resulted in a significant decline in immune performance. also there is specific information available on how HTR2A contributes to the immune response in trout [10]. As formerly indicated, genetic diversity and alterations in methylation of the HTR2A gene have been shown to play an essential role in several psychiatric illnesses. This reported serotonin (5-HT) can affect cytokine synthesis in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells by preventing the formation of tumor necrosis factor-alpha (TNF-alpha), a process mediated by the HTR2A receptor. In this context, a positive correlation has been indicated between the DNA methylation of the *HTR2A* gene and both inflammation and disease progress in rheumatoid arthritis. This proposes that elevated methylation of *HTR2A* is related to heightened inflammatory reactions and more hard disease signs in rheumatoid arthritis patients. In a cell culture medium test, the wound healing ability of LX-2 cells was investigated following therapy with various 5-HT2A receptor antagonists. This treatment followed by rapid apoptosis in these cells. Consistent with of the current trial, adding tryptophan into the diet of fish led to more potent outcomes following dip vaccination. This showed that tryptophan plays a role in elevating vaccination efficiency and enhancing vaccine success in aquaculture [11].

It has been indicated that fluoxetine reduce viral RNA reproduction, resulting in the preventing of human enterovirus B (HEV-B) and HEV-D replication. This result is related to fluoxetine's function as a selective serotonin reuptake inhibitor. In this context, HTR2A is reported to present an essential

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role. Administering SSRIs to mice with the HTR2A gene knocked out had no influence, explaining these mice staying significantly depressed.

HTR1B exhibits a role in regulating the serotonin system within lymphoid organs. [12]. The triggering of HTR1B is believed to affect the secretion of neurotransmitters that influence different physiological actions and behaviors in animals. These consist of the managing of prolactin, adrenocorticotropic hormone (ACTH), cortisol, and renin secretion, as well as governing food intake, body temperature manage, sexual behavior, and motor function. the HTR1B gene encoded The 5-HT1B receptor, that is a subtype of serotonin receptors and plays as a G-protein-coupled receptor (GPCR). It firstly controls serotonin secretion from terminals by functioning as an inhibitory auto-receptor. by starting of this receptor, signaling pathway begins in the post-synaptic membrane and extracellular signal-regulated kinase (ERK). controlling the production of Activity-regulated cytoskeleton-associated protein (Arc), is essential for Activated ERK which is largely found in postsynaptic membranes and exhibits an essential role in managing synaptic plasticity. This action emphasized the significance of 5-HT1B receptors in modulating neural actions related to mood controlling and other cognitive functions. [13]. dysregulation of synaptic plasticity plays a significant involvement in the pathophysiology of schizophrenia. This includes structural alterations such as lowered complexity in dendritic arbors and declined density of dendritic spines. These changes resulted in impaired neural connectivity and actions, which are hallmarks of the illness. besides, higher transcript levels of HTR1B have been seen in schizophrenia, implying further disturbance in neurotransmitter systems that may worsen the condition's signs. The complicated interact between these factors indicate the multifaceted nature of schizophrenia's neuropathological mechanisms [14].

applying the HTR1B antagonist GR-127935 via intraperitoneal administration to 24-week-old hens over five consecutive days led to an elevation in feather pecking and aggressive behavior between the birds. [15]. In an equivalent way, it was inferred that the expression of HTR1B in the brain was importantly decreased in White Leghorn poultry with high feather pecking behavior compared to those with few feather pecking behavior [16]. In highly malignant breast cancer types, there is a significant down-regulation of serotonin receptors consist of HTR2A, HTR2B, HTR2C, HTR4, HTR1A, HTR1B, and HTR7. This down-regulation is related to sever inflammation development in these cancers. The involvement of these serotonin receptors pointed out their essential task in modulating the immune microenvironment of breast cancer and influencing detection. This infers the involvement of the serotonin system in modulating and inhibiting inflammation [17]. In a mouse model, the pharmacological prevent of serotonin receptor HTR1B inhibited 5-HTP from inhibiting eosinophil migration through endothelial cells. This implies that both HTR1A and HTR1B

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receptors in endothelial cells (not eosinophils) play an essential role in modulating the anti-inflammatory effects of 5-HTP by preventing eosinophil in transendothelial migration, which is essential to prevent the development of allergic inflammation.

totally, THRs play a vital function in the immune response within the body's immune system. However, specific serotonin receptors like HTR1B and HTR2A have been shown to elevate in the spleen of layer chicks after vaccination against the H5N1 virus. While recent literature indicates the role of HTR2A upon confronting infectious agents, our findings indicate that serotonin receptor HTR2A is highly expressed in the spleen post-vaccination. This implies that controlling this receptor probably would be able to enhance vaccine outcomes in poultry.

Melatonin plays a wide range of biological functions. It is essential for advancing sleep and controlling seasonal rhythms and circadian. Additionally, melatonin interplay to act as an antioxidant, anti-aging processes, helps and inhibits excessive cell death (anti-apoptosis) and tumor formation (anti-tumorigenesis). It effectively offsets free radicals, which can destroy cells. additionally, melatonin affects the start of puberty and plays an essential role in modulating both adaptive immune and innate responses within the body. Melatonin's potent immune-regulatory properties have related to it with various autoimmune states, such as rheumatoid arthritis, and inflammatory bowel disease, systemic lupus erythematosus, type 1 diabetes mellitus, multiple sclerosis. in current study showed notable expression of the MTNR1B gene in the spleen of layer chickens following a double dose of an H5N1 virus vaccine. In separate animal studies, Drazen et al. suggested that inhibiting MTNR1B gene expression might be more crucial than inhibiting MTNR1A for increasing humoral immunity and melatonin-induced cell-mediated in mice. This infers that MTNR1B would be able to play a more significant role in immune responses compared to MTNR1A [18]. The MTNR1 receptor is widely dispersed specific lymphocytes such as CD4 and CD8 cells, entire of the immune system, including key organs like the spleen and thymus. The melatonin-MTNR pathway mediated a vital function in modulating immune reactions, particularly in influencing autoantibody production and controlling B cell activation. This indicates that melatonin's interaction with its receptors can have profound effects on different directions of immune regulation and response. Research has shown that melatonin intermediated a significant role in regressing tumor growth through multiple cancer types, including prostate, lung, breast and gastrointestinal cancers. In addition, findings have noted changes in the MTNR1B gene related to recurrent chordoma; specifically, deletions and down-regulation of this gene have been reported. Melatonin's anticancer effects are mostly mediated through its receptors like MTNR1B and involve mechanisms such as inhibiting cell migration and metastasis [19]. A study on chickens showed that addition of melatonin to the ration of young chickens decreased inflammation in the duodenum

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induced by LPS. This was mostly due to blocking the TLR4 signaling pathway, which resulted in decreased epithelial cell deaths and lower levels of inflammatory substances (IL-6, IL-4, Caspase3 and TNF-α) and enhanced intestinal immunity through increased immunoglobulin A and production of mucin 2 (MUC2) [20]. Additionally, laboratory experiments have indicated that melatonin increases the functionality of T lymphocytes in chickens. [21, 22]. The literature emphasized the importance of melatonin receptors in the immune system organs of poultry. Melatonin is necessary for stimulating lymphocyte multiplication, increasing intestinal immune barrier function and suppressing inflammatory reactions in poultry. Studying and understanding the melatonergic system would gain valuable findings that may increase vaccination outcomes in poultry.

MirRNAs

MicroRNAs (miRNAs) as non-coding RNAs are necessary for controlling gene expression. They impact various biological processes and the reaction after RNA transcription from DNA and before protein synthesis. Their functions in regulating gene expression, particularly in immune cell development and inflammation, are currently under investigation.

Six miRNAs associated with the HTR2A receptor has been shown significant gene expression in the spleen tissue of chicks. in different types of cancer MicroRNA-101 (miR-101) well-known as a tumor suppressor, it is expressed by two precursor transcripts, miR-101-1 and miR-101-2, which control oncogenic pathways and prevent tumor growth and progression. Throughout search for microRNAs (miRNAs) exhibited miR-101 as a powerful suppressor of autophagy that influenced autophagy in breast cancer cells. miR-101 is a abled agent of inhibiting both induced and basal forms of autophagy, including those triggered by rapamycin and etoposide recognized in this study [23]. A microRNA that plays a significant role in prostate cancer is miR-193b, its methylation leading to its down-regulation, which induce tumor progression. In an experiment miR-193b using pre-miR-193b oligonucleotides introduced in the 22Rv1 prostate cancer cell line resulted in vastly growth inhibition. probably miR-193b indicating its potential as a therapeutic target for controlling cancer growth and acting as an epigenetically quieted tumor suppressor in prostate cancer. miR-193b straightly aimed estrogen receptor-α (ERα), this is clear in breast cancer and repressed the estrogen-driven multiplication of breast cancer cells and gastric cancer. Repressor of hepatocellular carcinoma (HCC) is MiR-302c the play role by inhibiting the growth and advance of the disease. In comparison to normal and chemosensitive tissues, MiR-181a is significantly up-regulated in chemo-resistant ovarian cancer tissues [24]. miR-1575 and miR-1664 were recognized as up-

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regulated miRNAs in an experiment examining the expression of microRNAs in poultry lungs infected with the widely pathogenic avian influenza virus H5N1. This finding emphasized their potential roles in modulating immune responses or other biological processes during H5N1 infection. [25, 26]. These miRNAs mediate interactions between host organisms and their viruses, indicating their important role in regulating gene expression in response to viral infections in chicks [27]. In conclusion, our *in silico* analysis generates the hypothesis that an enhanced serotonergic system in the spleen is associated with the immune response to vaccination in poultry. Furthermore, the identified microRNAs (miRNAs) and their target gene *HTR2A* emerge from this study as candidate biomarkers worthy of further investigation for assessing post-vaccination immune status. This study is constrained by its bioinformatic nature. The conclusions are derived from secondary data analysis, which is an inherent limitation. Consequently, our findings are hypothesis-generating and must be validated through direct experimental approaches, including qRT-PCR or Western Blot, in subsequent studies.

5. Conclusions

In conclusion, our *in silico* analysis generates the hypothesis that an enhanced serotonergic system in the spleen is associated with the immune response to vaccination in poultry. Furthermore, the identified microRNAs (miRNAs) and their target gene *HTR2A* emerge from this study as candidate biomarkers worthy of further investigation for assessing post-vaccination immune status. This study is constrained by its bioinformatic nature. The conclusions are derived from secondary data analysis, which is an inherent limitation. Consequently, our findings are hypothesis-generating and must be validated through direct experimental approaches, including qRT-PCR or Western Blot, in subsequent studies.

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Authors' Contribution

Study concept and design: Y.B

Acquisition of data: A.B

Analysis and interpretation of data: A.B

Drafting of the manuscript: M.M, Y.B

Critical revision of the manuscript for important intellectual content: Y.B

Statistical analysis: A.B

Administrative, technical, and material support: Y.B

Ethics

In this study, we analyzed data sets freely available in the public domain of NCBI website. Accordingly, ethics committee approval was not required.

Conflict of Interest

The authors declare that they have no conflict of interest related to this study.

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