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Comparison of the Transcriptomic studies in indigenous chicken versus IBL-80 broiler chicken

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ABSTRACT

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Indigenous chicken breeds have played a vital role in contributing towards nutritional security and economic assistance to the rural people in the nation. These birds are well adapted to the local environment and possesses resistance to a number of diseases. However, they lack performance in terms of growth characteristics for which they are not prefered for raising in commercial broiler farming. A study was conducted to compare lung and trachea tissue transcriptomic analysis data for three groups of chickens namely Aseel, Punjab Brown, and IBL80 commercial broiler. RNA-sequencing results provided 5,282 differentially expressed genes (DEGs). Out of the total DEGs, 1478 from Aseel, were up-regulated and 930 were down-regulated. 1872 DEGs belonged to Punjab Brown in which 697 were up-regulated and 1175 down-regulated and IBL-80 exhibited 1931 DEGs but 714 were upregulated and 1217 downregulated. Interestingly, all three chicken groups revealed more downregulated DEGs. Gene Ontology (GO) enrichment analysis led to most of the comparable terms among the chicken groups. The count of genes related to innate immunity were greater in Aseel compared to Punjab Brown and commercial broiler. The majority of genes were chemokines or chemokine receptors. Aseel exhibited relatively higher count of chemokines and chemokines receptor-related genes compared to Punjab Brown, and IBL-80 commercial broiler.

Keywords: Aseel, Commercial broiler, Differentially expressed gene, Pathway analysis, Punjab Brown, Transcriptome

INTRODUCTION

The poultry industry plays a crucial role in the livestock sector, being the third-largest producer of eggs and the fourth-largest producer of chicken meat worldwide. The domesticated chicken (*Gallus gallus domesticus*) is one of the most important livestock species, serving as a key source of animal protein for human consumption. In India, poultry farming has steadily expanded, especially with the increasing demand for native chicken meat (Padhi *et al.*, 2022). The nation has officially identified 19 Indigenous chicken breeds (Niranjan *et al.*, 2022). Indigenous poultry birds are well-adapted to various climatic conditions (Dalal *et al.*, 2022) and exhibit a higher tolerance to elevated temperatures, making them more resilient to diseases compared to exotic or commercial broilers (Ramasamy *et al*., 2010). Aseel is one of the supreme indigenous chicken breeds of India. Aseel chickens can be located largely in Andhra Pradesh, Madhya Pradesh, Orissa, and Rajasthan. This breed is known for combativeness, magnificent gait, high endurance, and fighting skills (Dalal *et al*., 2022). The productivity is low in Aseel yet the birds are known for their meat quality with preferable taste and enriched flavour (Haunsh & Prince, 2021). On the contrary, Punjab Brown is a dual-purpose breed in the poultry industry and is reared for meat and egg production. The breed is mainly found in the northern states of Punjab and Haryana (Kanakachari *et al*., 2022.). The indigenous chicken breeds of India are known to be more disease-resistant than commercial breeds. The artificial selection performed for improvement in growth performance neglects the disease resistance parameters in commercial chicken (Sehrawat *et al*., 2021). Thus, commercial chicken remains immunologically deprived and indigenous chicken remains immunologically resilient to many diseases (Raj *et al.*, 2009). The immune system acts as the first line of defense against the infective pathogen. Both innate and adaptive immunities work supremely towards reorganization and clearance of pathogens. The more or less production of immune factors magnifies the pathophysiology of diseases (Prabhu *et al.*, 2021, Saini *et al.*, 2022)

RNA sequencing (RNA-seq) is a powerful data set that offers a snapshot insight into a particular tissue. Recently RNA-sequencing (RNA-seq) is performed using Next-generation sequencing (NGS) technologies (Mortazavi *et al*., 2008) The transcriptome displays a dynamic physiological picture of gene expression. Even more, transcriptomic profiling is efficient to investigate the comprehensive repertoire of genes related to various economic traits in poultry as well as in livestock species

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(Ahlawat *et al.*, 2021). Transcriptomic profiling technology is in increasing demand which offers to catalog and identify the differentially expressed genes expressed at a particular point of time in any organism, and comes with the potential to discover unique functional properties including disease resistance, growth, and environmental habituation (Wu *et al.*, 2020)

Among all the body systems in poultry, the respiratory tract is an important part to investigate because pathogens are frequently responsible to cause disease in birds. Their respiratory tract serves as the pathway for pathogens to enter the body system and regulate the magnitude of disease complexity. The most common and hazardous respiratory diseases in poultry are avian influenza (AI), Newcastle disease (ND), infectious bronchitis (IB), infectious laryngotracheitis (ILT), and Avian metapneumovirus (Islam *et al.*, 2022) Therefore, in this study, the differential gene expression profile of the lungs and trachea of Aseel, Punjab Brown, and commercial broiler (IBL-80) were compared by RNA-seq to get a comprehensive insight into identifying the key genes associated with stronger immune response in the defferent breeds of chicken.

MATERIALS AND METHODS

Ethics Statement

All experiments were carried out in compliance with the guidelines set by the Institutional Animal Ethics Committee (IAEC) of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU/2018/IAEC/47/09).

Experimental Design

Two-day-old chicks of Aseel, Punjab Brown, and Commercial broiler (IBL-80) were purchased from the state of Punjab. No vaccination was administered to the chicks. For 42 days, the experimental chickens were taken care of under standard hygienic conditions at the Poultry farms of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The chickens were kept under surveillance daily and had indispensable access to feed and water. On the 42nd day, the experimental chicken was slaughtered, and two tissues lung and trachea were collected. The tissues were immersed and preserved in RNA Later (Ambion, Austin, TX, USA) and maintained at 4°C for 24 hours, and thereafter at -80° C for subsequent use. Total RNA was obtained from both tissues (lung and trachea) using Trizol (Ambion, Austin, TX, USA). DNase I (Thermo Scientific) digestion was done post-RNA extraction. The RNA quantification was done to check the concentration and purity was determined by measuring absorbance at 260nm and A260/A280 ratio using a NanoDrop One (Thermo Scientific, USA). The purified RNA was preserved at -80°C up to needed for subsequent analysis.

RNA Sequencing and Data Analyses

The purified RNA from the samples were sent for sequencing to Agri Genome Labs Pvt. Ltd. (Kerala, India) for Next-generation sequencing on Illumina 150 bp Paired End (Nova Seq 6000). The raw sequenced data has been submitted to NCBI (National Centre for Biotechnology Information) under the short read archive. The fastq files were submitted to the SRA of NCBI (Bioproject: PRJNA972351, Biosample: SAMN35074231).

The Raw reads generated were subjected to removing the adapter and trimming low-quality bases using AdapterRemoval-v2 (version 2.3.1). The processed reads were further aligned to the *Gallus gallus* genome assembly (GCA_016699485.1). The differential expression analysis for the 8 samples was done using the cuffdiff program of the Cufflinks package. Differentially expressed genes (DEGs) were identified using R programming environment version 4.1.0. The gene with $\log_2$ fold-change ≥ 2.0 or ≥ -2.0 along with the p-value ≤ 0.05 were retained throughout the analysis. Further, constructed a heat map between the three experimental chickens illustrating the expression difference. Venn diagrams of overlapped DEGs were constructed using http://bioinformatics.psb.ugent.be/webtools/Venn/. between the three experimental chicken groups. The functional annotation and enrichment analysis of the pathways in DEGs was conducted using DAVID (Huang *et al.*, 2009), while the STRING database was utilized for further investigation of protein-protein interactions (PPI) among differentially expressed genes, applying default parameters (Szklarczyk *et al.*, 2016).

RESULTS AND DISCUSSION

RNA sequencing result

The average numbers of raw reads, processed reads, and aligned percentages in Aseel, Punjab Brown and Commercial Broiler are given in Table (1).

Differentially expressed genes and enriched pathways

The comparison of the transcript levels between the three chicken groups was done and DEGs meeting the criteria of $\log_2$ fold change greater than or equal to 2.0 or less than or equal to -2.0. along with the p-value ≤ 0.05 were identified. An overall count of 5,282 differentially expressed genes (DEGs) were observed from the three chicken groups. A total of 1,479 DEGs were observed in Aseel, while 1,872 and 1,931 DEGs were identified in Punjab Brown and IBL-80 commercial broilers, respectively, with 660 DEGs common to all three chicken groups. In Aseel out of the 1479 DEGs, 549 were upregulated and 930 were downregulated. The DEGs were subsequently explored for GO analysis in order to understand their functional enrichment. The top 10 gene ontology terms for the Aseel are shown in Table (2). The significantly enriched biological function was cell differentiation, cell adhesion, angiogenesis, immune system, and inflammatory responses. A fundamental part of the membrane, plasma membrane, extracellular space, and extracellular region were top terms for the cellular

Table 1: RNA sequencing summary data for Aseel, Punjab Brown, and Commercial Broiler with their respective Lung and Trachea tissues.

S.No.	Sample Name	Sample Name	Total read count	Read count after rRNA removal	Aligned percentage
1.	Aseel	Lung	54,523,434	54,059,248	94.63
2.		Trachea	50,821,690	50,821,690	93.94
3.	Punjab Brown	Lung	32,695,158	31,337,832	93.80
4.		Lung	34,892,244	34,234,960	93.63
5.		Trachea	40,342,628	39,864,188	93.51
6.		Trachea	30,712,610	30,251,374	92.65
7.	Commercial Broiler	Lung	59,699,960	59,205,720	95.59
8.		Trachea	59,650,598	39,170,430	91.03

component. The top terms for molecular functions were calcium ion binding, RNA polymerase II transcription factor activity, growth factor activity, actin filament binding, and cytokine activity.

For the 1872 DEGs in Punjab Brown, 697 were upregulated while 1175 were downregulated, the enriched gene ontology terms are depicted in Table (3). The major biological processes identified encompassed the control of transcription from the RNA polymerase II promoter, its enhancement, and cellular functions differentiation, cell adhesion, enhancement of gene expression, and immune responses. In the cellular component category, enrichment was observed in the integral membrane component, plasma membrane, extracellular space, and extracellular region. The predominant molecular functions involved RNA polymerase II transcription factor activity with sequence-specific DNA binding, sequence-specific DNA binding at the RNA polymerase II core promoter proximal region, calcium ion binding, and G-protein-coupled receptor affinity activity.

Furthermore, enrichment analysis using the PANTHER tool highlighted immune-related responses in Aseel, Punjab Brown, and Commercial Broiler, as illustrated in Figure 1. Among the 931 differentially expressed genes (DEGs) in commercial broiler, 714 were upregulated, while 1,217 were downregulated. Gene Ontology (GO) analysis for biological processes identified key functions such as cell-differentiation, cell-adhesion, positive modulation of gene expression, signaling of G-protein-coupled receptor, and suppression of apoptosis. The cellular component category included the integral membrane component, plasma membrane, extracellular space, and integral plasma membrane component. Lastly, in molecular functions, the most enriched GO terms were calcium ion binding, RNA polymerase II transcription factor function, DNA binding with sequence specificity, and actin filament interaction, and cytokine activity, as presented in Table 2 to Table 4.

Enriched Immune-related DEGs

The immune-related DEGs were also explored using the PANTHER tool. PANTHER enrichment analysis revealed 29 DEGs in Aseel associated with immune responses. And similarly, there were 30 and 39 DEGs associated with immune-related responses in Punjab Brown and commercial broiler respectively. Venn diagram (Figure 2) was constructed to analyze the unique elements in each experimental chicken group. Amongst the three groups, 5 genes were common including

(*IL7R, ZAP70, BST1, CRTAM, and VEGFC*). Surprisingly 16 genes were unique to Aseel including (*THEMIS, CST7, EOMES, CCR8, CXCL13L3, FRK, BLK, CCL1, GATA3, CXCL13L2, VEGFD, CCL5, CXCL13, STAP1, CXCR5, and CD1*), Punjab Brown represented 12 unique genes having (*CD180 SYK DYSF CD79B LY96 EFNA2 CCR6 HELLS FLT3 CCL4 CX3CR1 LAT2*), while only 8 genes were unique to commercial broiler including (*LCK, CCL17, CCR7, BANK1, FES, RELA, AvBD14, and ANXA1*).

Gene network analysis

The interaction among the proteins encoded by DE immune-related genes were explored through STRING (Figure 3). For Aseel the unique genes were carried out to explore the protein-protein interaction resulted in PPI enrichment p-value 1.8≥-06. The annotation information of these proteins was provided and KEGG analysis showed 1 pathway of cytokine- cytokine receptor interaction pathway whereas Reactome analysis showed chemokine receptor bind cytokine. Similarly, for the Punjab Brown and commercial broiler the unique genes related to immune responses were studied to explore protein-protein interactions (PPI).

PPI enrichment had a significant association with Punjab Brown (p-value 6.93≥-08). In biological processes, the gene ontology enrichment and annotation information of the proteins revealed macrophage activation implicated in immunological responses, leukocyte Intercellular adhesion, B lymphocyte receptor signaling pathway, and neutrophil chemotaxis. It

Table 2: Gene ontology terms for Differently expressed genes with higher expression in Aseel

Category Aseel	Terms	Number of genes	P- Value
Biological	Cell differentiation	34	0.015703981
Process	Cell adhesion	28	0.070821657
	Positive regulation of gene expression	23	0.001103749
	Negative regulation of apoptotic process	22	2.01E-08
	Extracellular matrix organization	16	0.001316166
	Angiogenesis	16	1.48E-08
	Immune response	16	0.047320148
	Inflammatory response	15	0.032618284
	Wnt signaling pathway	14	0.027869193
	Neuron differentiation	13	0.037343346
Cellular	Integral component of membrane	205	7.56E-04
Component	Plasma membrane	151	1.82E-07
	Extracellular space	122	7.18E-21
	Extracellular region	73	2.61E-12
	Integral component of plasma membrane	58	3.01E-04
	Cell surface	30	2.08E-05
	Synapse	29	0.003220001
	Neuron projection	27	6.81E-05
	Extracellular matrix	25	1.67E-06
	Dendrite	22	0.01416244
Molecular	Calcium ion binding	63	8.41E-05
Functions	RNA polymerase II transcription factor activity, sequence-specific DNA binding	55	0.006603603
	Growth factor activity	26	1.18E-07
	Actin filament binding	23	0.002943803
	Cytokine activity	21	0.014563451
	Receptor binding	17	0.039972134
	RNA polymerase II regulatory region sequence-specific DNA binding	16	1.73E-04
	Calmodulin binding	14	2.51E-04
	Heme binding	12	6.70E-04
	Iron ion binding	12	0.012299711

(fold change ≥ 2.0 or ≥ -2.0; p-value ≤0.05)

Table 3: Gene ontology terms for differently expressed genes with higher expression in Punjab Brown (PB)

Category PB	Terms	Number of genes	*P* Value
Biological	Regulation of transcription from RNA polymerase II promoter	87	0.015703981
Process	Positive regulation of transcription from RNA polymerase II promoter	53	0.070821657
	Cell differentiation	44	0.001103749
	Cell adhesion	41	2.01E-08
	Positive regulation of gene expression	36	0.001316166
	Immune response	34	1.48E-08
	Positive regulation of transcription, DNA-templated	28	0.047320148
	Positive regulation of cell proliferation	27	0.032618284
	Negative regulation of apoptotic process	26	0.027869193
	G-protein coupled receptor signaling pathway	24	0.037343346
Cellular	Integral component of membrane	297	5.64E-11
Component	Plasma membrane	204	5.47E-04
	Extracellular space	164	1.79E-05
	Extracellular region	100	0.06876925

	Integral component of plasma membrane	85	6.52E-07
	Extracellular matrix	37	0.006934009
	Cell surface	37	0.033447239
	Synapse	33	0.007957547
	External side of plasma membrane	32	4.21E-06
	Dendrite	28	0.011709836
Molecular	RNA polymerase II transcription factor activity, sequence-specific	83	8.41E-05
Functions	DNA binding		
	RNA polymerase II core promoter proximal region sequence-specific	73	0.006603603
	DNA binding		
	Calcium ion binding	69	1.18E-07
	G-protein coupled receptor activity	38	0.002943803
	Transcriptional activator activity, RNA polymerase II transcription	35	0.014563451
	regulatory region sequence-specific binding		
	Transcription factor activity, sequence-specific DNA binding	28	0.039972134
	actin filament binding	27	1.73E-04
	Growth factor activity	27	2.51E-04
	Cytokine activity	24	6.70E-04
	Receptor binding	21	0.012299711

(fold change ≥2.0 or ≥-2.0; p-value ≤0.05)

Table 4: Gene ontology terms for differently expressed genes with higher expression in Commercial Broiler (CB)

Category CB	**Terms**	**Number of genes**	***P* Value**
Biological	cell differentiation	41	0.004006601
Process	cell adhesion	38	3.33E-07
	positive regulation of gene expression	29	0.050046652
	G-protein coupled receptor signaling pathway	29	9.42E-04
	negative regulation of apoptotic process	28	0.006086793
	positive regulation of transcription, DNA-templated	26	0.089902286
	angiogenesis	23	1.99E-04
	heart development	19	0.001587833
	inflammatory response	19	0.051168634
	Wnt signaling pathway	17	0.009741761
Cellular	integral component of membrane	100	8.27E-05
Component	plasma membrane	45	4.80E-12
	extracellular space	39	4.17E-19
	integral component of plasma membrane	26	2.20E-07
	extracellular region	18	4.55E-07
	synapse	14	0.007193313
	cell surface	13	2.11E-04
	extracellular matrix	12	1.37E-07
	neuron projection	125	9.47E-04
	external side of plasma membrane	25	0.012791029
Molecular	calcium ion binding	78	5.54E-12
Functions	RNA polymerase II transcription factor activity, sequence-specific		
	DNA binding	71	0.008676056
	actin filament binding	26	2.64E-04
	sequence-specific DNA binding	22	0.009984102
	cytokine activity	21	0.005760943
	growth factor activity	20	0.037047591
	receptor binding	18	0.061982693
	iron ion binding	15	0.015386205
	calmodulin binding	14	0.026106811

(fold change ≥ 2.0 or ≥ -2.0 ; p-value ≤0.05)

demonstrated C-C chemokine binding, C-C chemokine receptor activity, and cytokine receptor function in Molecular function. Pathway analysis revealed only Reactome analysis with chemokine receptors binds chemokines. The PPI enrichment p-value was 0.282, and gene ontology enrichment was detected demonstrating monocyte chemotaxis, cell chemotaxis, and control of leukocyte activation for biological processes in commercial broilers.

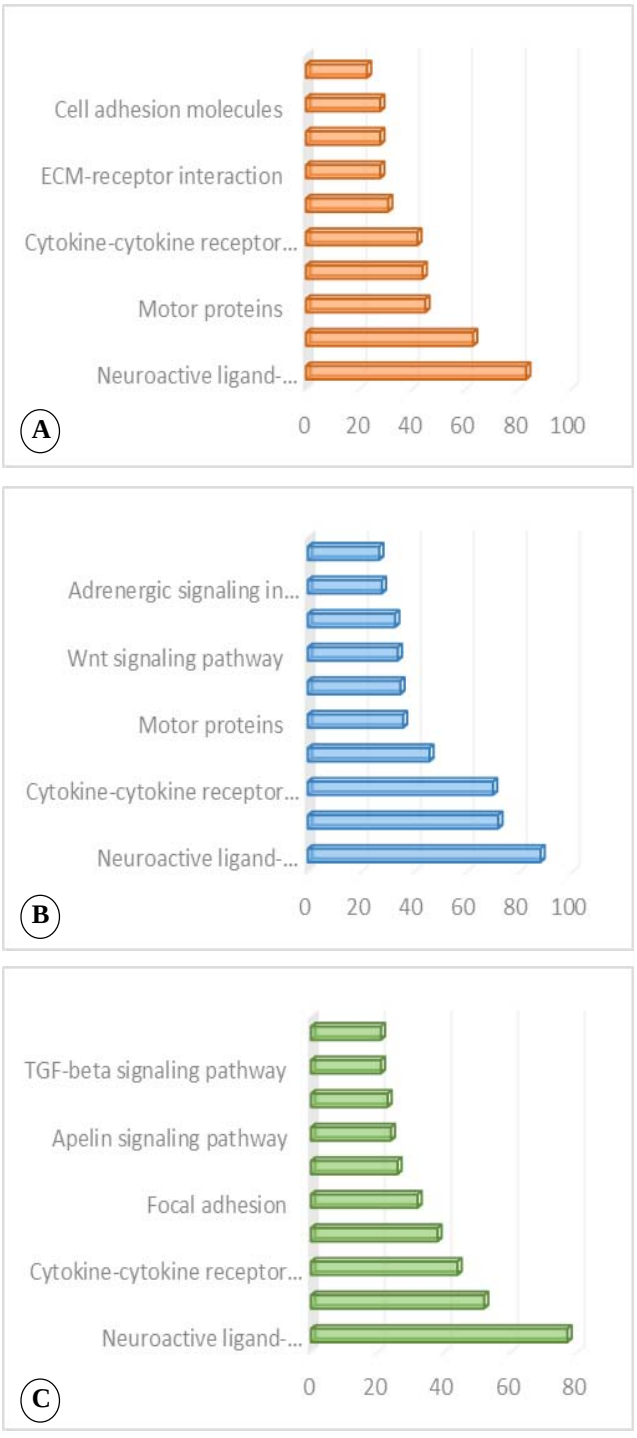

Fig. 1: Immune response terms associated with differently expressed genes in (A) Aseel, (B) Punjab Brown, and (C) Commercial Broiler chickens

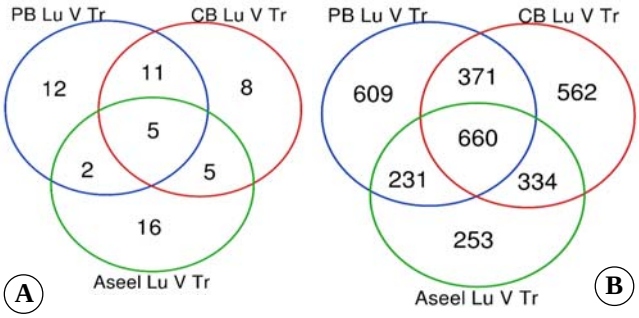

Fig. 2: Venn diagram illustrating differentially expressed genes. (A) Representation of differentially expressed genes between the three groups of chicken. (B) Venn diagram of PANTHER screened immune response-related genes.

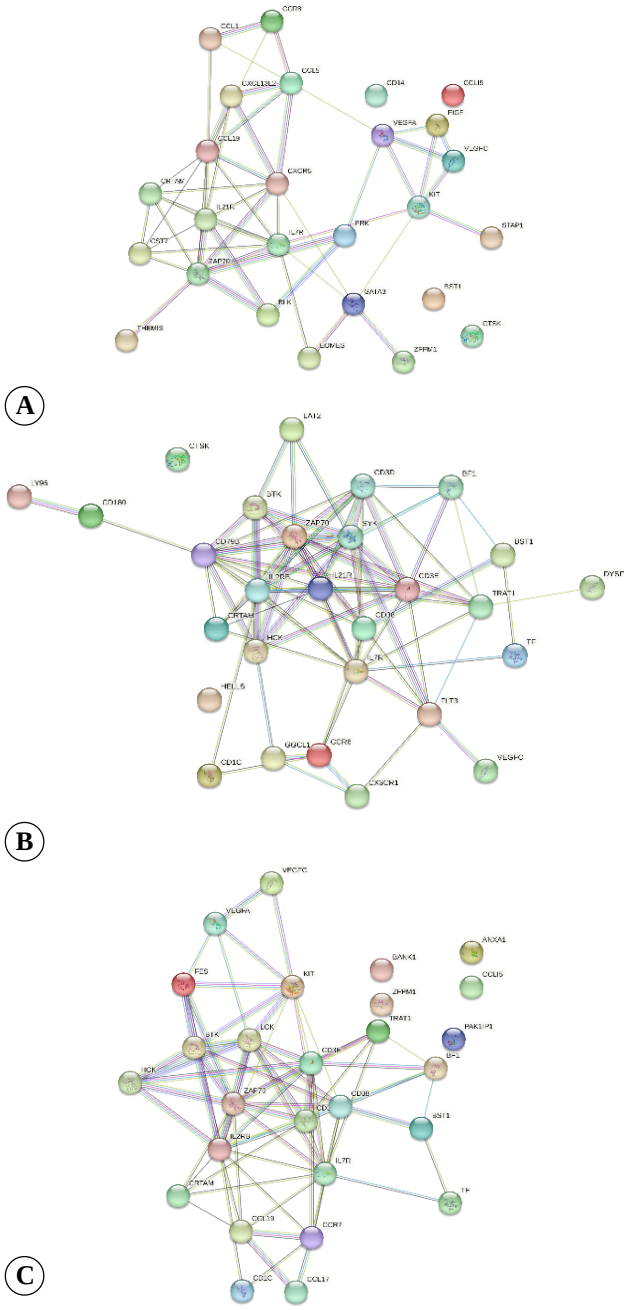

Fig. 3: Gene network analysis of immune response-related genes on (A) Aseel, (B) Punjab Brown and, (C) Commercial Broiler

In this study, we have carried out the transcriptomic profiling based on RNA sequencing of Lung and trachea tissues of Aseel, Punjab Brown breeds, and Commercial Broiler (IBL-80) strain of India. The chicken groups were taken into study under non-vaccinated conditions to explore the immune-related response generated in the chicken. The DEGs were identified using an R programming environment with the $\log_2$ fold change ≥ 2.0 or ≥ -2.0 along with the p-value ≤ 0.05. A total of 5,282 DEGs were identified from the three experimental chicken groups, of which 1479 DEGs were from Aseel, 1872 were from Punjab Brown, and 1931 were from Commercial Broiler. Keeping in view that commercial broiler showed the highest number of DEGs and Aseel showed the lowest. Moreover, 549 DEGs were upregulated and 930 were downregulated in Aseel, the 697 DEGs were upregulated while 1175 were downregulated in Punjab Brown, and for Commercial Broiler 714 DEGs were upregulated and 1217 were downregulated. Upon Venn diagram construction we revealed that among the three chicken groups, 660 DEGs were common in the three groups.

Based on the GO annotation data (generated using the DAVID tool) top 10 terms from biological processes, cellular components, and molecular functions were identified that in Aseel. Most of the DEGs participate in cell differentiation, cell adhesion, plasma membrane, calcium ion binding, immune response, cytokine activity, inflammatory responses. Given the conserved nature of innate immune responses across different organisms, additional genes related to innate immunity were identified using the PANTHER tool. 28 DEGs were screened to be related to immune responses. Venn diagram was constructed which highlighted 16 genes out of 28 including (*THEMIS, CST7, EOMES, CCR8, CXCL13L3, FRK, BLK, CCL1, GATA3, CXCL13L2, VEGFD, CCL5, CXCL13, STAP1, CXCR5, and CD14*). *CCR8, CCL1,* and *CCL5* were up-regulated chemokines. *CXCL13L, CXCL13L2 CXCL13, and CXCR5* were upregulated chemokine receptors.

Apart from the chemokines and chemokine receptors, there were other important innate immunity genes that were upregulated including *THEMIS* which is known as an important T-cell protein implicated in the activation of mature T cells and homeostasis (Liu *et al*., 2022). *CST7* is a leukocystatin that is found to play a role in immune regulation by inhibiting unique targets in the hematopoietic systems (Halfon *et al*., 1998).

1872 DEGs were found in Punjab Brown, out of which 697 were upregulated while 1175 were down-regulated, GO analysis revealed, most of the DEGs were involved in the regulation of transcription from RNA polymerase II promoter, positive regulation of transcription from RNA polymerase II promoter, cell differentiation, cell adhesion, positive regulation of gene expression and immune responses, an integral component of membrane, plasma membrane, extracellular space, RNA polymerase II transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific DNA binding, Calcium-ion binding, and G-coupled receptor binding activity. PANTHER tool was used to screen the immune response which results in 30 genes. Further, 12 genes were found as unique to Punjab Brown including (*CD180, SYK, DYSF, CD79B, LY96, EFNA2, CCR6, HELLS, FLT3, CCL4, CX3CR1, and LAT2*). *CCR6* and *CCL4* are chemokines and *CX3CR1* is chemokine receptor. In Commercial Broiler, 714 DEGs were elevated, whereas 1217 were downregulated. Commercial Broiler also represented GO enrichment as an integral component of the membrane, plasma membrane, and extracellular space as the former two chicken positive regulation of gene expression, G-protein coupled receptor signaling pathway, and negative regulation of the apoptotic process, a calcium ion binding, RNA polymerase II transcription factor activity, sequences-specific DNA binding, actin filament binding, and cytokine activity. When Commercial Broiler was examined for immunological responses using the PANTHER tool, 8 distinct genes were discovered, including *(LCK, CCL17, CCR7, BANK1, FES, RELA, AvBD14,* and *ANXA1*). Only the two chemokine genes *CCL17* and *CCR7* are presented. *BANK1* has been identified as a possible positive regulator of B cell signaling (Georg *et al*., 2020). There were 5 (*IL7R, ZAP70, BST1, CRTAM, and VEGFC*) innate-immune genes that were common in three chicken groups.

CONCLUSION

The comparative analysis of the expression profile between three distinct breeds/lines (viz. Aseel, Punjab brown, and commercial broiler) indicated that there was no distinct variation between the breeds. The gene expression was found to be comparable with each other. Further studies are warranted with larger population size to determine any genetic pattern in the expression of module genes linked to economically significant traits in chickens.

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Author Contribution

JS: DEGs identification, pathway analysis, and manuscript writing; PVS: Collection of chicks, RNA extraction; YS: Collection of chicks, RNA extraction; Shakti Dash Kant management inputs and simpling; CSM designed the study and supervised the work. All authors read and approved the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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