Bioinformatics Analysis of the Microarray Data of Broiler Chicken Spleen Vaccinated with ND Vaccine Genotype VII and Challenged with *Clostridium Perfringens* Type A

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Abstract

Necrotic enteritis is one of the most deadliest illnesses that affect most species of birds including broiler that developed due to infection by pathogenic strain of Clostridium perfringens, most of its pathogenesis caused by NetB and TpeL toxins Therefore, the current study was designed to define most genes, microRNA and different molecular and biological pathways implicated in the pathogenesis of NE. Differentially expressed genes obtained from GEO database (GSE13085) by applying GEO2R analysis. ShinnyGo 8.0 software were applied to identify the set of possible diseases associated with the obtained DEGs and Protein-protein interaction (PPI) network obtained from String database and visualized by Cytoscape software. microRNAs that regulate most of DEGs obtained from Network Analyst. We found that top 5 upregulated hub genes were TLR-4, STAT-1, TRAF-6, Casp-8, and cxcl-12, top 3 downregulated hub genes were CD4, BCL-2, and CD44. Additionally toll like- receptor signaling pathway and cytokine- cytokine receptor interaction was the most implicated pathways NE. furthermore gga-mir-143 is microRNA that regulate most important DEGs. Based on our finding we can suggest that TLR-4, Casp-8, CD4, BCL-2, and gga-mir-143 represent a novel therapeutic target for NE induced immunosuppressive effect.

Keywords: Bioinformatics analysis, microarray data, broiler chicken spleen, ND vaccine genotype VII

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1-introduction

An estimated yearly monetary loss of about six billion dollars is incurred by the broiler business worldwide due to the debilitating disease known as necrotic enteritis (NE), which affects several species of birds, including broiler chickens (Wade et al., 2015). The characteristics of the

multifactorial intestinal disease known as necrosis, several foci of bleeding, and inflammation in the mucosa layer of the hens' small intestine are produced by the growth of pathogenic Clostridium perfringens (CP) (Van Immerseel et al., 2009).

CP has the ability to form sporulation, strictly anaerobic, Gram-positive bacteria that has wide distribution in food, earth, and the digestive system of both healthy and sick animals and people (Kiu & Hall, 2018). CP has capacity to produce heat-resistant endospores in unfavorable environmental circumstances is another trait that accounts for the observation of the bacterium's extreme persistence throughout nature (Hustá et al., 2023).

Death rates from 2 to 50% are associated with NE in chickens that are 2 to 6 weeks old. This disease categorized into clinical and subclinical form (Broom, 2017). Poultry growers can easily identify the clinical form since it causes acute disease that ultimately ends in bird death. while the sub-clinical type of the disease is less evident as it correlated with decrease in feed transformation efficiency and gut microflora imbalance instead of a significant surge in fatalities (Moore, 2023).

An abundance of diet- and environment-related risk factors, such as a nutrition high in non-starch polymers and raw protein, high moisture content, high stocking density, inadequate air flow, and bad litter situation, as well as other stressors that can weaken chicken immunity and upset the delicate balance of intestinal microbiota, contribute to the overgrowth of CP (Alizadeh et al., 2021; Moore, 2016).

Typically, CP is categorized into seven types of toxins (from A to G) based on the toxins it produces, which include beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE), alpha (CPA), and NE B-like toxin NetB. The primary causal toxinotypes of NE in poultry production are thought to be C. perfringens type A (generating CPA), C (producing CPA and CPB), and G (producing CPA and NetB) (Qi et al., 2023).

A significant part of the pathophysiology of NE is played by NetB. It is a pore-forming toxin that is plasmid-encoded and unique to CP isolated from poultry affected by NE. It is similar to S. aureus alpha-hemolysin. It creates heptagonal holes on the membranes of its target cells (Keyburn et al., 2008; Yan et al., 2013).

A toxin belonging to the vast clostridial toxin family is called TpeL., is one example of a potential NE pathogen. It is suggested that C. perfringens type A/G strains augment the NE pathogenicity through the TpeL toxin (Gu et al., 2019). It contains several domains with glycosyltransferase activity, activity of autocatalysis, and a transmembrane domain that transports the enzyme into the host cell's cytoplasm (Chen & McClane, 2015). It inhibits Ras signaling and triggers cell apoptosis by mono-glycosylating Ras proteins and self-mediating host cell entrance (Guttenberg et al., 2012).

VR-10B is the locus that we previously discovered to be common in isolates of CP that cause NE (Lepp et al., 2013). It, when expressed, encodes an adhesive sortase-dependent pilus (NE pilus) necessary for NE pathogenesis and collagen binding(Lepp et al., 2021). Seven open-reading frames (ORFs) make up the VR-10B locus, which encodes 3 pilin subunits (CnaA, FimA, and FimB), a signal peptidase, an enzyme called sortase, and a possible 2 component regulatory system (TCS) (Lepp et al., 2013).

TCS listed here as the PilS sensor histidine kinase (SK) and PilR response regulator (RR), together known as PilRS. Virulent C. perfringens strains that have had their cnaA, fimA, and fimB disrupted no longer produce NE pilus, which significantly attenuates their virulence and reduces their capacity to bind collagen (Wade et al., 2015). The accessory gene regulator (Agr)-like quorum sensing (QS) system may positively control the VR-10B operon, according to a prior publication (Yu et al., 2017).

The expressions of Toll-like receptors (TLR-4), (TLR-2), Nuclear factor kappa-B(NF-κB), Janus kinase (JAK3), and Signal transducer and activator of transcription (STAT6) were all elevated with exposure to CP. The lack of a discernible impact of C. perfringens exposure on MyD88 expression may suggest that TLRs trigger NF-κB via signaling pathways independent of MyD88 (Tang et al., 2022). Through MyD88-dependent or independent signaling pathways, TLRs can set off subsequent inflammatory responses. These pathways activate NF-κB, which in turn causes the production of pro-inflammatory mediators such as Tumor necrosis factor alpha (TNF-α),
Interleukin 1 beta(IL-1β), Interleukin 6(IL-6), IL-8, and Inducible nitric oxide synthase (iNOS) (Pasare & Medzhitov, 2004).

2- Material and method

2.1 Microarray data

The gene expression dataset was analyzed to detect DEGs after being obtained from the Gene Expression Omnibus (GEO) database (GSE13085). Data involved two distinct groups non medicated group (N=12 Gallus gallus) and medicated one (N=11 Gallus gallus). The standard parameters for detecting differentially expressed genes (DEGs) are *P*-value < 0.05 and fold change (log2 FC > 1.5).

2.2 Gene ontology and Enrichment Analyses pathways

Gene Ontology (GO) is a popular *in silico* tool that Offers extensive details on the gene function of certain genomic products based on predefined parameters. This analysis is divided into three parts: molecular functions (MF), cellular components (CC), and biological processes (BP). Higher-level understanding of biological processes and instruments is facilitated by the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. The DAVID database (https://david.ncifcrf.gov/), CLueGo – Cytoscape plugin application, and ShinnyGo 0.8 online software (http://bioinformatics.sdstate.edu/go80/) provide a wide range of functional annotation tools to researchers so they analyze and understand the biological importance of certain gene lists. We analyze GO and KEGG analysis of DEGs with false discovery rate (FDR) less than 0.05.

2.3 Protein-protein interaction (PPI) network building and hub gene analysis

To retrieve interacting genes, DEGs were uploaded to the Search Tool (STRING, https://string-db.org/),a database of predicted and known protein-protein interactions, to explore the relationships amongst the proteins that the detected DEGs encode. Cytoscape was used to provide results with a lowest possible interaction rating of 0.4. The PPI network was also searched for hub genes using the maximal clique centrality (MCC) approach and CytoHubba, a Cytoscape plugin

application that provides an easy-to-use interface for investigating important nodes in biological networks. The highly interconnected proteins and molecular pathways found in PPI string analysis are finely grouped in MCODE, another Cytoscape plugin application, in a well-defined molecular pathway that contributes to the development of NE.

2.4 Detection of the most contributed gene for all pathways implicated in NE.

With Network Analyst (https://www.networkanalyst.ca/), the majority of the contributing gene that developed NE was found. this stage was carried out by uploading the acquired hub genes with their fold change acquired from GEO2R analysis for the DEGs. This finds gene–protein and/or transcription factor interaction.

2.5 Detection of the expected microRNA contributes to NE.

The top 250 differentially expressed genes was submitted to the online ShinnyGo 8.0 program (http://bioinformatics.sdstate.edu/go80/). In addition to validating KEGG pathways, enrichment analysis, and gene ontology (BP, CC, and MF), this program may identify microRNA - the molecular processes involved in the interaction between DEGs - and pinpoint the chromosomal locations of DEGs.

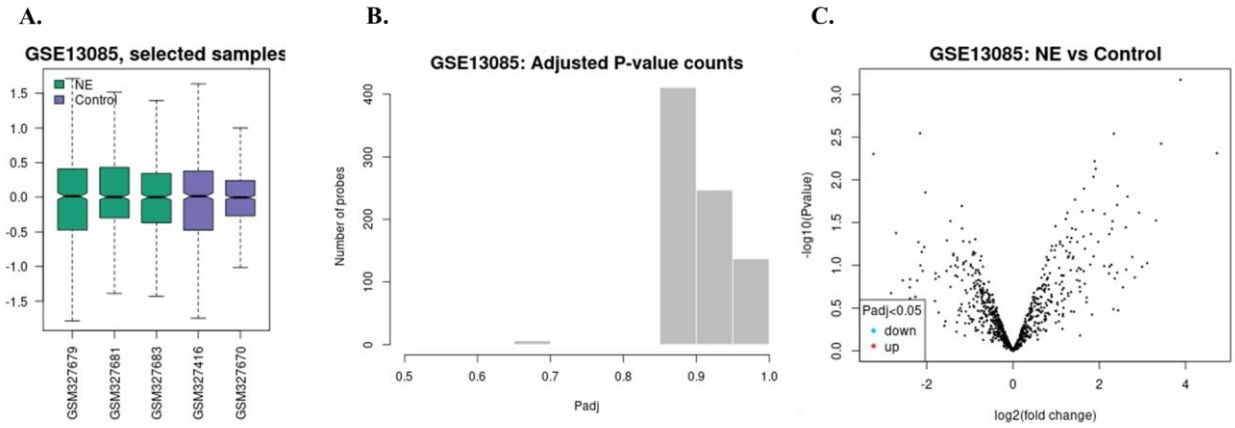
2.6 Detection of the DEGs and disease interaction

ShinnyGo 8.0 online software was used to identify the set of possible diseases associated with the obtained DEGs and PPI string analysis (http://bioinformatics.sdstate.edu/go80/) to confirm the role that the DEGs played in the emergence of NE and its associated disease.

3-Result

3.1 differentially expressed genes (DEGs).

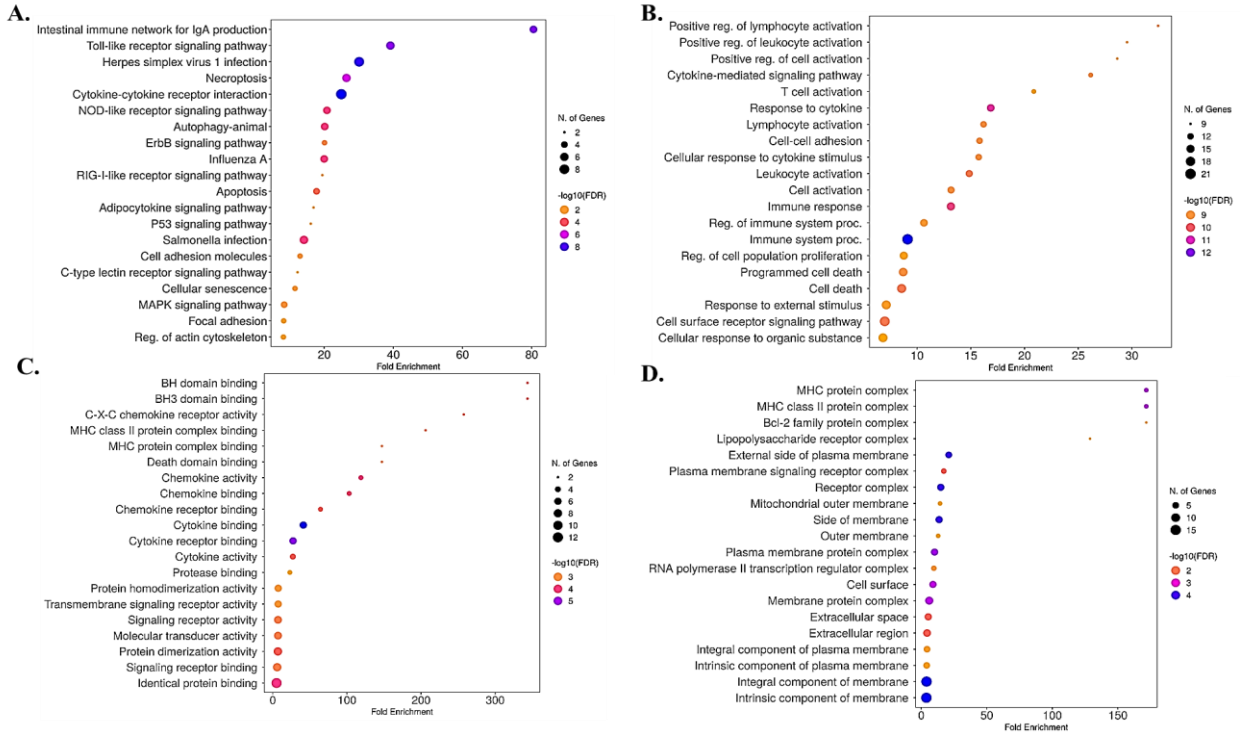
After comparing the GEO2R analysis of control and NE, we take top 250 differentially expressed genes, from which 117 displaying downregulation and 133 displaying upregulation. (Fig. 1 A. – C.).



(Fig. 1) GEO2R reveal DEG with adjusted *P*-value < 0.05 and fold change (log2 FC > 1.5). A. samples (NE, control), B. adjusted p-value for the obtained genes, and C. volcano plot of the DEG

3.2 Functional Enrichment Analysis of DEGs

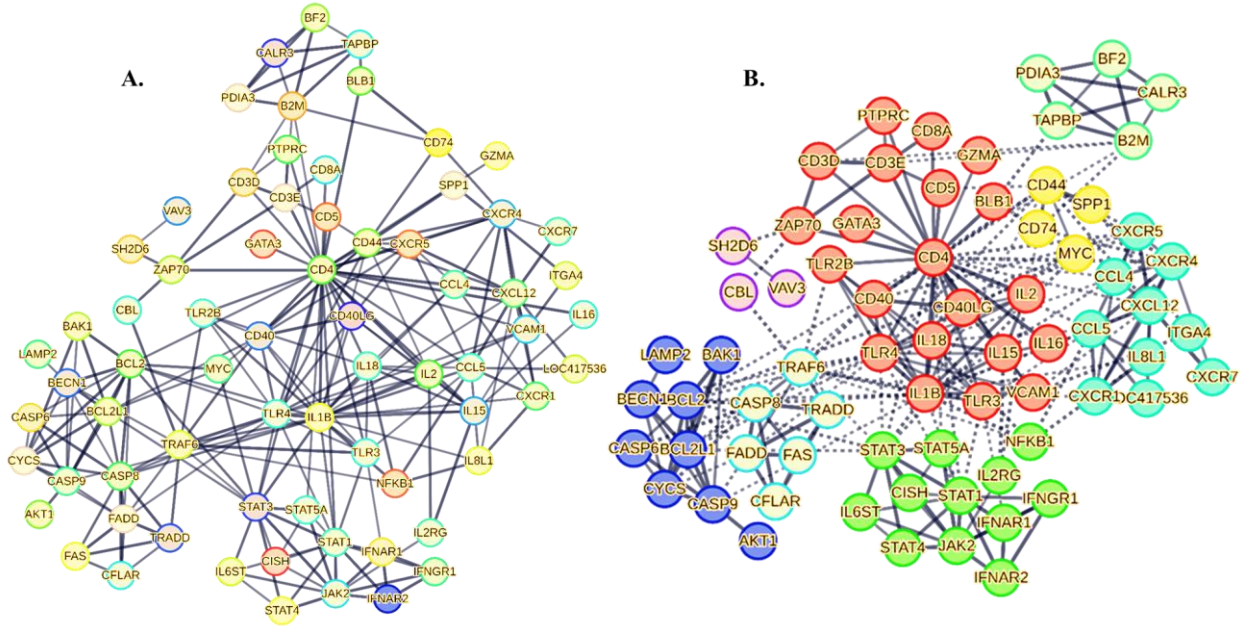
To identify different roles of the detected DEGs gene ontology (GO), such as molecular function (MF), cellular components (CC), biological process (BP), and KEGG pathway. The KEGG pathways analysis exhibited intestinal immune network for IgA production, toll like- receptor signaling pathway, herbes simplex virus 1 infection, necroptosis, cytokine- cytokine receptor interaction, NOD-like receptor signaling pathway, autophagy animal, ErbB signaling pathway, influenza A, RIG-like receptor signaling pathway, apoptosis, adipocytokine signaling pathway, p53 signaling pathway, salmonella infection, cell adhesion molecule, c-type lectin receptor signaling pathway, cellular senescence, MAPK signaling pathway, focal adhesion, Reg of actin cytoskeleton(Fig. 2A). In addition, The GO result for BP are positive reg. of lymphocyte activation, positive reg. of leukocyte activation, positive reg. of cell activation, T cell activation, response to cytokine, lymphocyte activation, cell-cell adhesion, cellular response to cytokine stimulus, leukocyte activation, cell activation, immune response, Reg. of immune system proc, immune system proc, Reg. of cell population proliferation, programmed cell death, cell death, response to external stimulus, cell surface receptor signaling pathway, cellular response to organic substance (Fig. 2B).Furthermore, GO analysis for molecular function (MF) mentioned as BH domain binding, BH 3 domain binding, C-X-C -chemokine receptor activity, MHC class II protein complex binding, MHC protein complex binding, death domain binding, chemokine activity, chemokine binding, chemokine receptor binding , cytokine binding, cytokine receptor binding, cytokine activity, protease binding, protein homodimerization activity, transmembrane signaling receptor activity, signaling receptor activity, molecular transducer activity, protein dimerization activity, signaling receptor binding, identical protein binding(Fig. 2C). Finally, GO analysis for cellular components (CC) include MHC protein complex, MHC class II protein complex, Bcl-2 family protein complex, lipopolysaccharide receptor complex, external side of plasma membrane, plasma membrane signaling receptor complex, receptor complex, mitochondrial outer membrane, side of membrane, outer membrane, plasma membrane protein complex, RNA polymerase II transcription regulator complex, cell surface, membrane protein complex, extracellular space, extracellular region, integral component of plasma membrane, extrinsic component of plasma membrane, integral part of the membrane, external part of the membrane(Fig. 2D).



(Fig. 2) GO and KEGG database identify functional enrichment analysis of DEGs (A. - D.). A. KEGG pathways include intestinal immune network for IgA production, toll like- receptor signaling pathway, herbes simplex virus 1 infection, necroptosis, cytokine- cytokine receptor interaction, NOD-like receptor signaling pathway, autophagy animal, ErbB signaling pathway, influenza A, RIG-like receptor signaling pathway, apoptosis, adipocytokine signaling pathway, p53 signaling pathway, salmonella infection, cell adhesion molecule, c-type lectin receptor signaling pathway, cellular senescence, MAPK signaling pathway, focal adhesion, Reg of actin cytoskeleton, B. BP include positive reg. of lymphocyte activation, positive reg. of leukocyte activation, positive reg. of cell activation, T cell activation, response to cytokine, lymphocyte activation, cell-cell adhesion, cellular response to cytokine stimulus, leukocyte activation, cell activation, immune response, Reg. of immune system proc, immune system proc, Reg. of cell population proliferation, programmed cell death, cell death, response to external stimulus, cell surface receptor signaling pathway, cellular response to organic substance, C. MF include BH domain binding, BH 3 domain binding, C-X-C -chemokine receptor activity, MHC class II protein complex binding, MHC protein complex binding, death domain binding, chemokine activity, chemokine binding, chemokine receptor binding , cytokine binding, cytokine receptor binding, cytokine activity, protease binding, protein homodimerization activity, transmembrane signaling receptor activity, signaling receptor activity, molecular transducer activity, protein dimerization activity, signaling receptor binding, identical protein binding, and D. CC include MHC protein complex, MHC class II protein complex, Bcl-2 family protein complex, lipopolysaccharide receptor complex, external side of plasma membrane, plasma membrane signaling receptor complex, receptor complex, mitochondrial outer membrane, side of membrane, outer membrane, plasma membrane protein complex, RNAP II transcription regulator complex, cell surface, membrane protein complex, extracellular space, extracellular region, integral component of plasma membrane, extrinsic component of plasma membrane, integral component of membrane, extrinsic component of membrane.

3.3-PPI

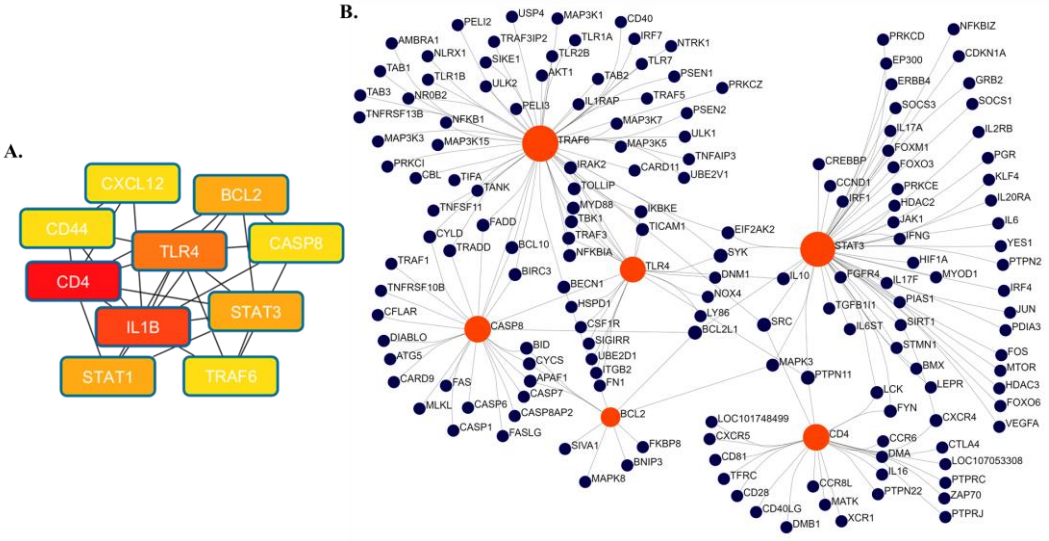
PPI is obtained from string data base with medium confidence of 0.400 (Fig. 3A) and clustering of genes into eight cluster mentioned as CD4 (TLR2B, CD40, CD40LG, IL2, TLR4, IL18, IL15, IL16, IL1B, TLR3, VCAM1,PTPRC, CD3D, CD3E,CD8A,GZMA,CD5,BLB1,GATA3, and ZAP60), BF2 (PDIA3, TAPBP, B2M, and CALR3), CXCL12 (CCL4, CCL5,CXCR5,CXCR4,TGA4,CXCR7,IL8L1,CXCR1, and C417536), CD44 (CD74,MYC, and SPP1), JAK-STAT1 (CISH, STAT3, STAT5A, IL6ST, STAT4, IFNAR2, IFNAR1,IFNGR1,IL2RG, and NFKB1),FAS (FADD,CASP8,TRADD,CFLAR, andTRAF6) and BAK1-BCL2L1 (LAMP2,BCL2,BECN,CASP6,CYCS,CASP9, and AKT1)(Fig. 3B)



(Fig. 3) protein -protein interaction that encode differentially expressed genes and clustering of them into eight cluster (A. & B.). A. PPI and B. clustering of genes into eight cluster mentioned as CD4, BF2, CXCL12, CD44, JAK-STAT1, FAS, and BAK1-BCL2L1.

3.4- Hub genes and transcription factor regulation

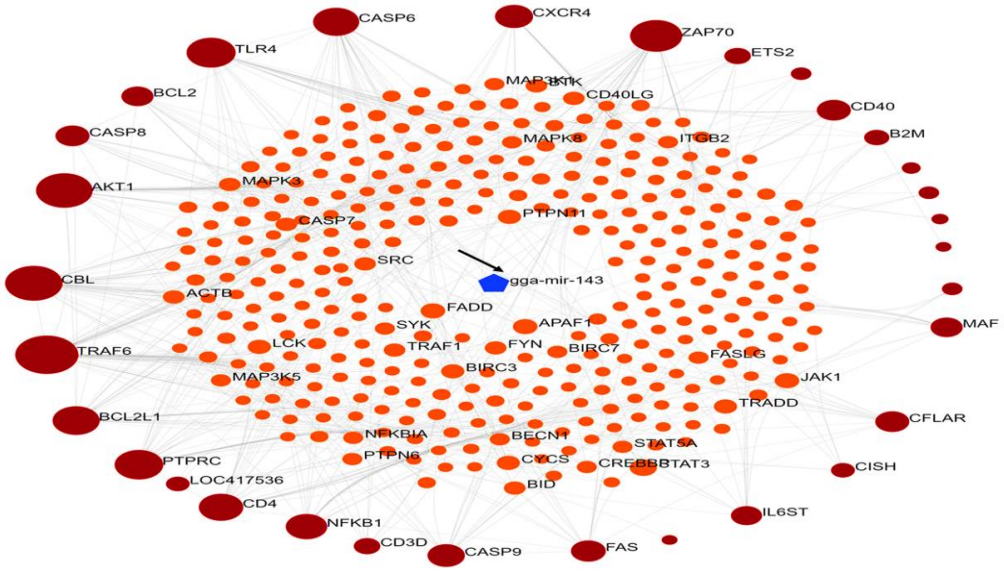
Top ten hub genes are STAT3, stat1, TRAF6, TLR4, BCL2, CASP8, and CD4, CD44, IL1B, and CXCL12 (Fig. 4A) and transcription factors that regulated it (Fig. 4B)



(Fig. 4) top ten hub genes and the transcription factors regulate their function (A. & B.). A. Top ten hub genes and B. top ten hub genes-transcription factor interaction.

3.5- microRNA regulated DEGs

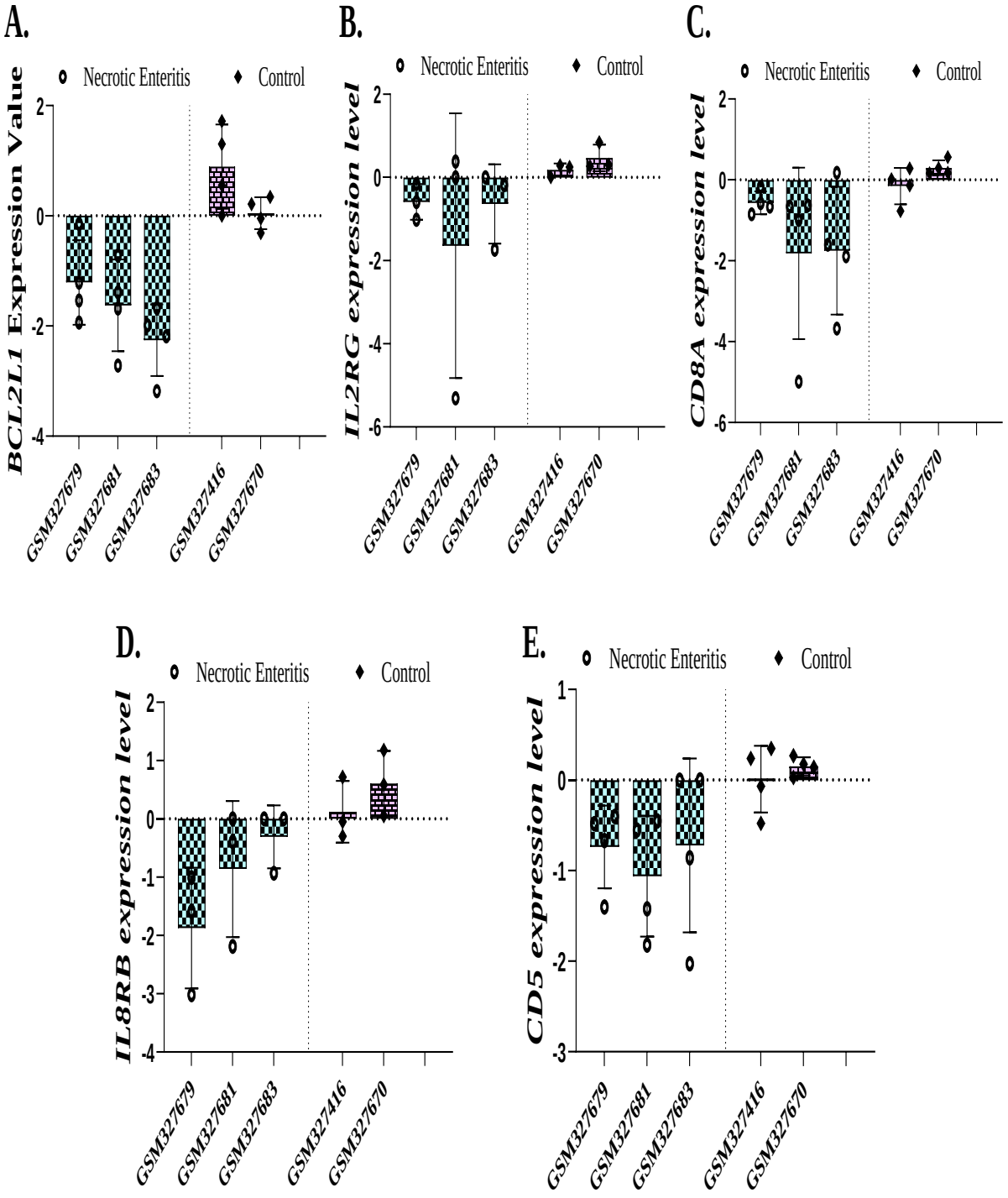
We found that gga-mir-143 is microRNA that regulate most important DEGs by applying Network Analyst (Fig. 5).



(Fig. 5) Network Analyst reveal that gga-mir-143 regulate most important DEGs

3.6-Top five downregulated genes

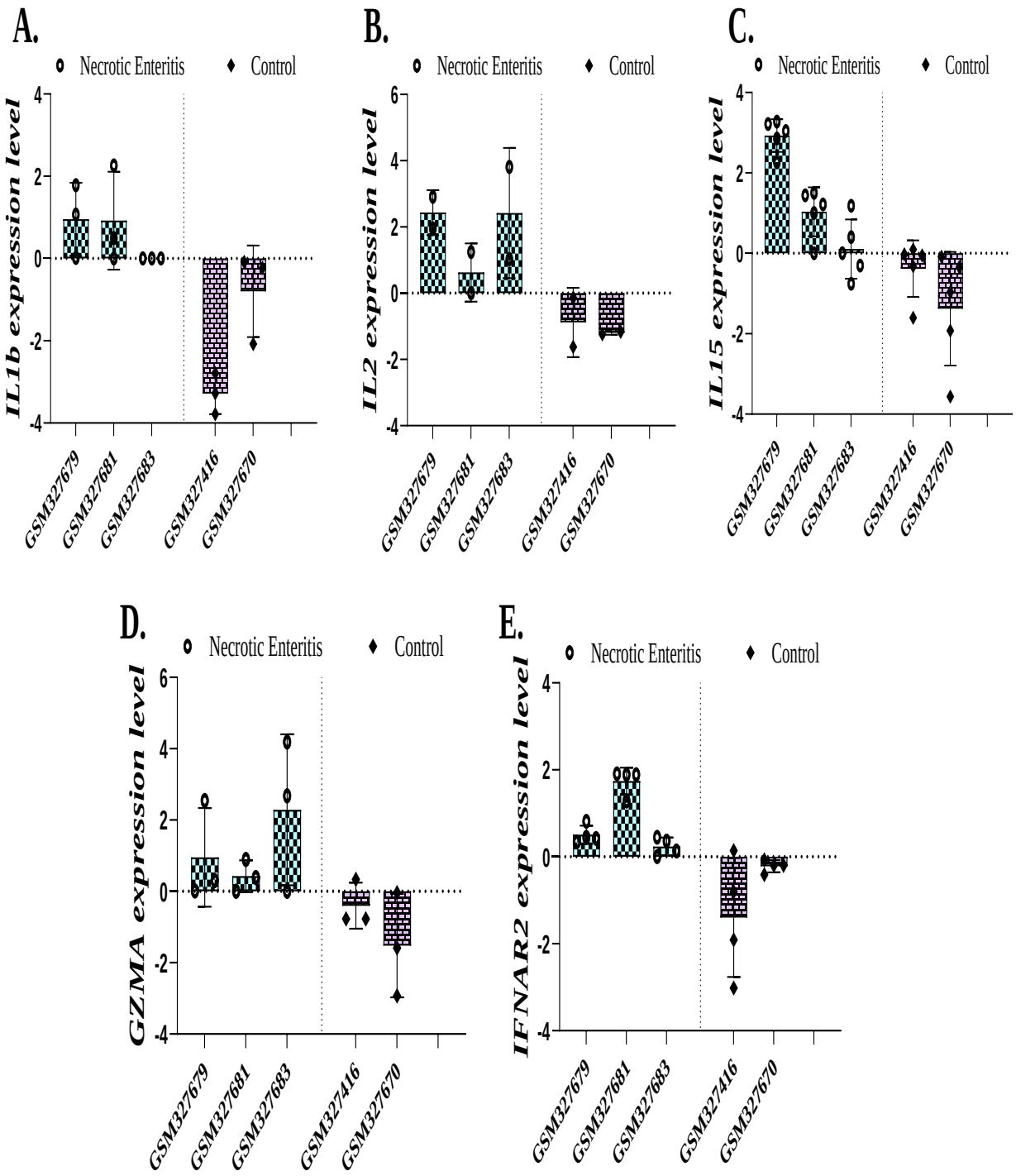
Bioinformatic analysis indicated that top 5 downregulated genes between NE and control group are BCL2L1, IL2RG, CD8A, IL8RB, and CD5 (Fig. 6 A. - E.) & (Fig. 9D.)



(Fig. 6) expression pattern of top five downregulated genes between NE group and control one (A. – E.). A. BCL2L1, B. IL2RG, C. CD8A, D. IL8RB, and E. CD 5.

3.7- Top five upregulated genes

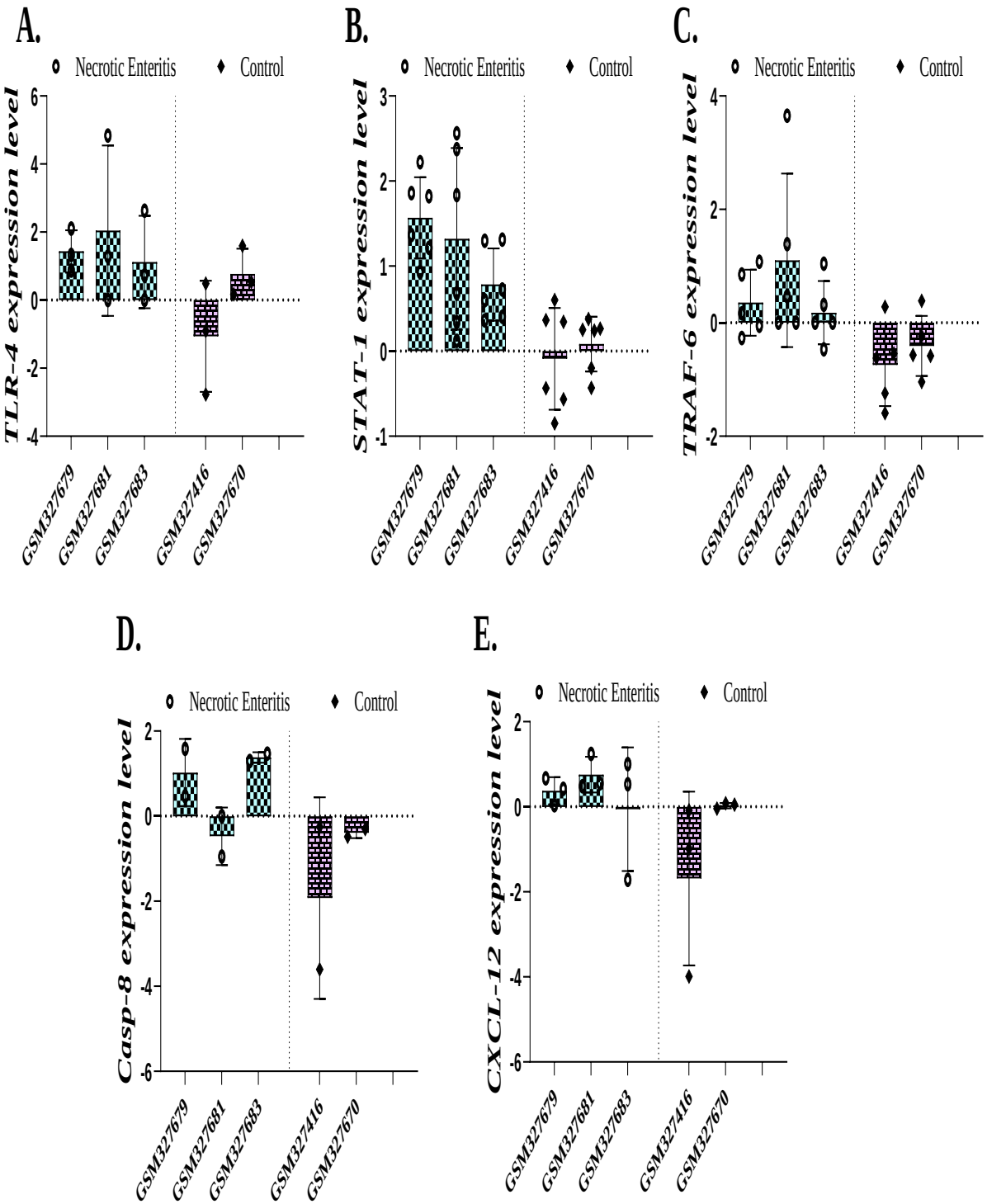
The top 5 genes that were upregulated between the NE and control groups, according to bioinformatic analysis, are IL1b, IL2, IL15, GZMA, and IFNAR2(Fig. 7 A. - E.) & (Fig. 9D.).



(Fig. 7) expression pattern of top five upregulated genes between NE group and control one (A. – E.). A. IL1b, B. IL2, C. IL15, D. GZMA, and E. TFNAR2.

3.8-Top five upregulated hub genes.

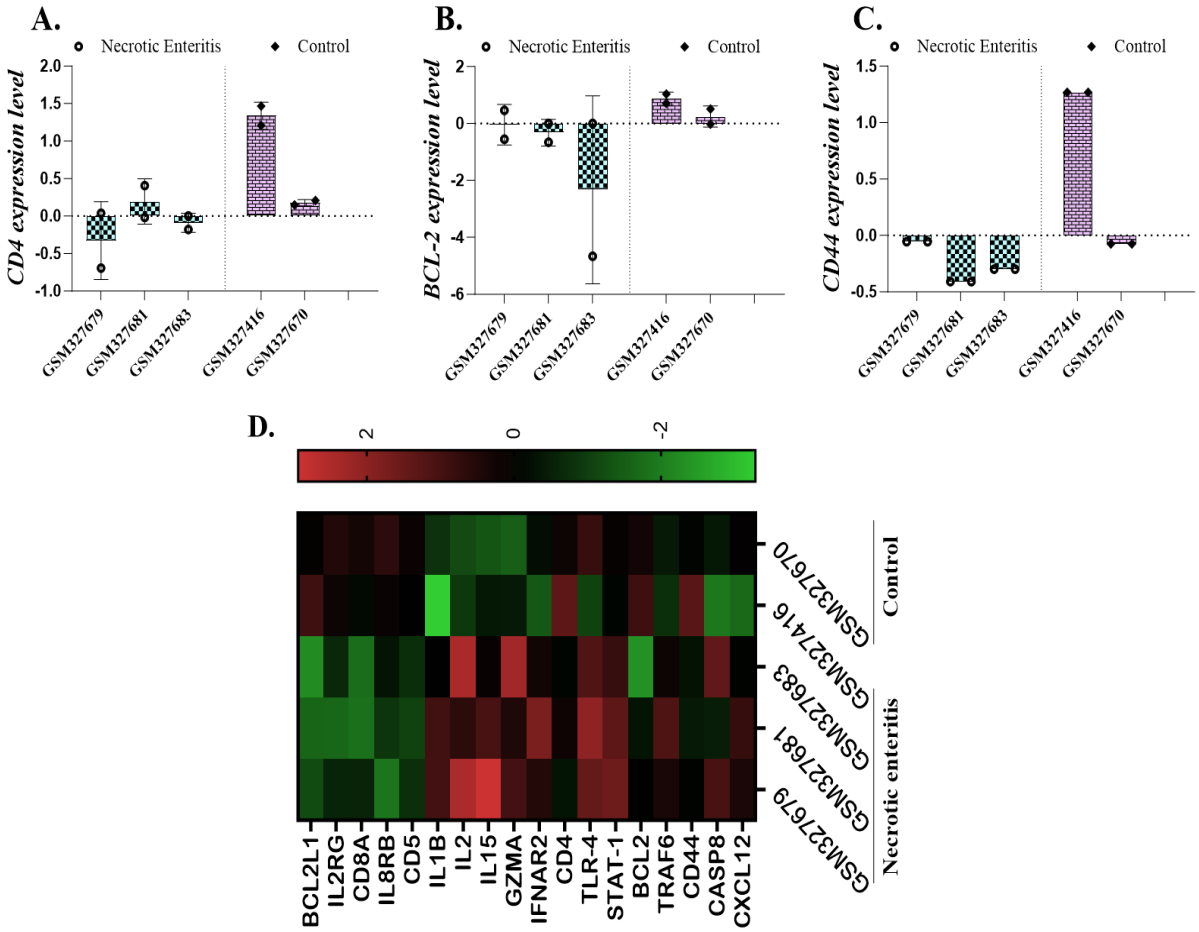
Bioinformatic analysis reveal TLR-4, STAT-1, TRAF-6, Casp-8, and cxcl-12 are the top 5 upregulated hub genes in NE group vs control one (Fig. 8 A. - E.) & (Fig. 9D.).



(Fig. 8) expression pattern of top five upregulated hub genes between NE group and control one (A. – E.). A. TLR-4, B. STAT-1, C. TRAF-6, D. Casp-8, and E. CXCL-12.

3.9-Top three downregulated hub genes.

Bioinformatic analysis showed that top 3 downregulated hub genes in NE group and control group are CD4, BCL-2, and CD44 (Fig. 9 A. - E.) & (Fig. 9D.).



(Fig. 9) expression pattern of top three downregulated hub genes between NE group and control one (A. – C.). A. CD4, B. BCL2, C. CD44.

4-Disccution

NE is one of the most devastating diseases [that affect broiler chicken at 2-6 weeks old and cause economic loss of about US$ 6 billion, Inflammation, many localized hemorrhages, and necrosis in the mucosa layer of the hens' small intestine are the hallmarks of the NE and this pathogenesis caused by clostridia perfringens toxins as NetB and TpeL. Several molecular pathways were implicated in the evolution and the pathogenesis of the disease, however the exact molecular Epigenetic pathways still elusive, thus this current study was designed to investigate DEGs, hub genes and hub miRNA that potentially contributed in NE onset and pathophysiology. The result of current in *silico* study reveal that the most important KEEG pathways that involved in the development of NE are intestinal immune network for IgA production, toll like- receptor signaling pathway, necroptosis, cytokine- cytokine receptor interaction, NOD-like receptor signaling pathway, autophagy animal, ErbB, RIG-like receptor signaling pathways, apoptosis, cell adhesion molecule, c-type lectin receptor signaling pathway, cellular senescence, MAPK signaling pathway, focal adhesion, Reg of actin cytoskeleton this pathways corresponded with (Fathima et al., 2022; Tang et al., 2022; X. Zhang et al., 2023; Zhao et al., 2021, 2022). In addition, BP that regulated by DEGs include positive reg. of lymphocyte activation, positive reg. of leukocyte activation, positive reg. of cell activation, T cell activation, response to cytokine that agreed with

(Daneshmand et al., 2022), moreover , MF include BH domain binding, BH 3 domain binding, C-X-C -chemokine receptor activity, MHC class II protein complex binding, MHC protein complex binding that in the line with (Miska et al., 2022; Zhao et al., 2022), furthermore , CC include MHC protein complex, MHC class II protein complex, Bcl-2 family protein complex, lipopolysaccharide receptor complex, the plasma membrane's outside that in accordance with (Daneshmand et al., 2022; Emami et al., 2020; Guo et al., 2021). The most critical cluster in the PPI of the DEGs are CD4, JAK-STAT1, CXCL12, BECN, and BAK-BCL2L1 (Shah et al., 2023; Yang et al., 2023), finally we hypothesis that gga-mir-143 is the most important hub miRNA that regulate DEGs (Jebessa et al., 2024).bioinformatics analysis reveal that top five hub upregulated and top three downregulated genes between NE group and control one are (TLR-4, STAT-1, TRAF-6, Casp-8, and CXCL-12) that in agree with (Gharib-Naseri et al., 2021; Tang et al., 2022; Woznicki et al., 2021), and (CD4, BCL-2, and CD44) (Gaghan et al., 2023) that in the line with (He et al., 2023; Yuan et al., 2024) respectively. In silico analysis reveal illustrate hub genes-transcription factors interaction as 1- CASP8 regulated by CASP7, CASP1, CASP6, FAS, FASL, TRAF1. TNFR1-induced apoptosis and caspase-8 activation (Siegmund et al., 2023). 2-BCL2 regulated by MAPK8, CASP8, and BAX. Erythropoietin protects against excessive apoptosis in the intestinal epithelium by reducing the overall number of ileal epithelial cells positive for caspase-3 and increasing Bcl-2 expression via the MAPK/ERK pathway (Subramanian et al., 2020). 3-TLR4 regulated by NOX4, EIF2, NFKB, MYD88, and HSPD. One of the most prevalent bacterial endotoxins in the gut, lipopolysaccharide (LPS) can cause macrophages to aggregate by activating TLR4. Additionally, it stimulates myeloid differentiation factor 88 (MYD88), which stimulates nuclear factor κB (NF-κB) and tumor necrosis factor receptor-related factor 6 (TNFR-related factor 6). Interactions between these variables cause cytokines and chemokines to be secreted more quickly, which speeds up the development of intestinal inflammation (Chang et al., 2024). Finally, JAK-STAT 3 pathway regulated by CREBB, CCND1, FOXO3, SOX1, HIF, TGFB, FGFR4, and SIRT1. As key mechanism for both apoptosis and cytokine response is Janus kinase 2/signal transducers and activators of transcription (JAK2/STAT). STAT-3 is a crucial factor in the transmission of inflammatory cytokine signals to the nucleus., it also regulates the anti-apoptotic B-cell lymphoma-extralarge (Bcl-xL) and B-cell lymphoma-2 (Bcl-2) genes, which helps to prevent apoptosis (Z. Zhang et al., 2023).

5-conclution

According to our bioinformatic analysis we can concluded that most implicated pathways in NE pathogenesis were toll like- receptor signaling pathway, cytokine- cytokine receptor interaction, and positive reg. of lymphocyte activation. Furthermore, TLR-4, Casp-8, CD4, BCL-2, and gga-mir-143 represent a novel therapeutic target for NE induced immunosuppressive effect. Further experimental studies are required to validate and confirm the current findings and searching prompt therapeutic and prophylactic interventions to burden the disease prevalence.

No Conflict of interest.

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