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Unraveling the immune response in Gaddi dogs: transcriptomic profiling of CpG-stimulated PBMCs

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ABSTRACT

The Gaddi dog, an indigenous breed from the Himalayan region, demonstrates remarkable adaptability to the region's rugged terrain and climate. Characterized by its cognitive abilities, agility, and innate loyalty, the breed is well-suited for herding and guarding, displaying evolutionary adaptations that enable its survival and functionality in mountainous environments. This study aimed to investigate the differential transcriptomic profile of peripheral blood mononuclear cells (PBMCs) in Gaddi dogs in response to CpG oligodeoxynucleotide (ODN), a ligand for Toll-like receptor 9 (TLR-9), to elucidate the immune responses associated with bacterial infections. PBMCs from Gaddi dogs were stimulated with the TLR-9 ligand, and RNA sequencing was performed using the Illumina NovaSeq 6000 platform. The expression data were analyzed using various bioinformatics tools. The differentially expressed genes (DEGs) were identified based on a log fold change (logFC) threshold of ≥3 or ≤-3, with a significance level of p-value ≤ 0.05. Functional enrichment analyses were conducted using Gene Ontology (GO) and pathway databases such as KEGG, Reactome, and PANTHER to explore the biological processes and pathways involved. The transcriptomic analysis revealed 1,072 DEGs, with 506 genes upregulated and 566 downregulated. The enriched pathways were associated with transcriptional regulation, immune modulation, cell survival, and stress responses, including autophagy, apoptosis regulation, viral and bacterial infection, and inflammation. The findings indicated a finely tuned regulation of immune responses in the Gaddi dog, highlighting breed-specific adaptations. This study represents the first transcriptomic analysis of immune responses in the Gaddi dog, providing a foundation for further research into its genetic potential and its ecological role within the Himalayan ecosystem.

Keywords: Apoptosis, CpG ODN, Gaddi dogs, Immune responses, PANTHER, Transcriptome

India hosts a rich and diverse spectrum of domestic animal breeds, including various canine breeds, many of which are indigenous and have developed specific adaptations tailored to distinct regions and functional roles (Vani *et al.* 2022). The Gaddi dog, an indigenous breed originating from the Himalayan region, is also referred to as the Himalayan Mastiff, Himalayan Sheepdog, Himalayan Hound, Himalayan Mahidant, Pahari Leopard Hound, or Gaddi Shepherd (Raja *et al.* 2017). Gaddi dog plays a crucial role in herding and safeguarding their livestock. These dogs are highly regarded for their ability to protect encampments and livestock from predators in the Himalayan region, serving as vital links between humans and their herds. The Gaddi dog is believed to share genetic ancestry with the Tibetan Mastiff, however, evolutionary processes, including geographical distribution and functional adaptations, have resulted in the development of its unique traits. Notably, the Gaddi dog differs from the Tibetan Mastiff by its longer legs, higher-set hocks, leaner build, and remarkable agility (Sankhyan *et al.* 2022). The Gaddi dog is known for its independent and fearless nature (Web ref a), thus exhibiting signs of diversifying selection in genes related to reproduction, immunity, and chemosensory perception, indicating adaptations for independent survival (Pilot *et al.* 2016). Gaddi dogs, are renowned for their resilience, high immunity, and disease resistance, adaptations that are crucial for their role as livestock guardians. Their robust health, coupled with a thick double coat and muscular build, enables them to withstand cold, harsh conditions and effectively protect herds against predators like leopards and bears. This high immunity and disease resistance make them ideal for enduring the harsh climates and rugged terrains of northern India (Web ref b). Preserving and safeguarding these dogs and their unique genetic traits is essential to ensure their continued presence and contribution to the cultural and agricultural landscape of the Himalayan region (Chaudhary *et al.* 2021).

Therefore, for understanding the Gaddi dog's physiological and immunological traits transcriptomic

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studies are important as they provide comprehensive insights into the gene expression profiles and molecular mechanisms. The present study was designed to explore the differential transcriptomic profile of Gaddi dog PBMCs challenged with CpG ODN (TLR-9 ligand) aiming to elucidate the immune-related responses of the Gaddi dog to bacterial infections.

MATERIALS AND METHODS

Ethical permissions and sample collection: Sample collection of peripheral blood from exotic and indigenous dogs was approved as per the reference number (GADVASU/2022/IAEC/64/11) dated 11th March 2022 of the Institutional Animal Ethics Committee. Blood samples of Gaddi dogs were collected from Palampur, Himachal Pradesh. The due permission was taken from the Director of Animal Husbandry, Himachal Pradesh for the collection of peripheral blood samples of Gaddi dogs (Reference number AHY/EPST(9)/86 Vol. VI10805; Dated: 06th June 2023). The blood samples were processed at the Department of Veterinary Microbiology in Dr. G.C. Negi College of Veterinary and Animal Sciences, Chaudhary Sarwan Kumar, Himachal Pradesh Krishi Vishvidyalaya, Palampur. Permission to this effect was obtained from the Dean, DGCN CoVAS (No. QSD.DCOVAS-CSKHPKV/VII-58(GA)/8048-49, dated 31/10/23).

PBMCs extraction and CpG ODN simulations: Initially, peripheral blood samples (3-5 ml) were collected aseptically from the cephalic vein of 8 two male and adult (age- 6-7 years) Gaddi dogs during the same season, using 500 µl of 0.5M EDTA as an anticoagulant. The samples were screened for Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC) to confirm the health status of the dogs. Based on the normal TLC and DLC reports, only two samples were further processed. The PBMCs from these two samples were isolated using Hisep™ LSM (Himedia) through density gradient centrifugation at 500 × g for 30 minutes, after which the PBMC layer (buffy coat) was carefully separated from the other blood components. The cells were washed with 1X phosphate-buffered saline, pH 7.4 at 500 × g for 10 minutes. The viability of the cells was assessed using the trypan blue dye exclusion method and ~2× 10^6 PBMCs were harvested in 6 well plates after counting by a hemocytometer. Isolated PBMCs were harvested with RPMI-1640 (HiMedia), with 10% FBS (HiMedia) and 1% penicillin-streptomycin (Thermos Fisher Scientific) at 37°C in a 5% CO_2 incubator. At the time of harvesting, the PBMCs were simulated with TLR-9 ligand i.e. Cytidine monophosphate guanosine oligodeoxynucleotides (CPG ODN) at 10 µg/ml for 12 hours (Singh *et al.* 2016). All protocols were performed under sterile conditions to avoid endotoxin contamination; replicate samples were processed identically.

RNA isolation and library construction: RNA was extracted from the control and treatment PBMCs, using the TRIzol method, and the quantity of RNA was determined using the Qubit® 4.0 fluorometer (Thermos Fisher Scientific), and its quality was assessed through an examination on a 1% agarose gel. The paired-end sequencing library was prepared using the KAPA mRNA HyperPrep Kit for Illumina (KAPA Biosystems). The amplified libraries were assessed at Unipath Specialty Laboratory Ltd on the TapeStation 4150 (Agilent Technologies) using HSD100 ScreenTape®.

RNA sequencing and bioinformatics analysis: The libraries prepared were subjected to the Illumina Nova-6000 platform for NGS to generate 2×150 bp paired-end reads**.** Sequencing and initial Bioinformatic analysis were performed by Unipath Specialty Laboratory Ltd in Ahmedabad, Gujarat. The raw sequencing data underwent preprocessing, including adapter removal and filtering out low-quality bases using Trimgalore (version 0.6.4) (Sun, 2020). The reads were aligned to a reference genome using the STAR aligner (version 2.7.10a) (Dobin *et al.* 2013). Transcript assembly was then conducted using StringTie (version 2.2.1) (Pertea *et al.* 2015; Kovaka *et al.* 2019). The abundances of the transcripts in all samples were estimated using StringTie, aided by merged transcripts generated from the merge stringtie step. A Python program (prepDE.py) was utilized to extract the read count information directly from the files generated by StringTie. Differential gene expression was inferred between control and treatment samples by using the R package edgeR (Robinson *et al.* 2010), a Bioconductor package based on the negative binomial distribution method. A comprehensive DEG analysis for functional enrichment was conducted, focusing on DEGs with logFC ≥ 3 or ≤ -3 and a p-value < 0.05. Gene Ontology (GO) analysis of the DEGs was conducted using the DAVID database (david.ncifcrf.gov/) to explore their functions and associated biological processes. To identify the signaling pathways linked to these DEGs, analyses were performed using KEGG (www.genome.jp/kegg/pathway.html), Reactome (reactome.org/userguide/pathway-browser), and PANTHER(http://pantherdb.org/) (Li *et al.* 2024). The STRING database (https://string-db.org/) was used to construct a protein-protein interaction (PPI) network for the DEGs (Szklarczyk *et al.* 2023).

qRT- PCR: Gene expression was validated using the qRT-PCR approach. From the differential expression data for mRNA-Seq, top 10 dysregulated genes were identified and analyzed for functionally enriched DEGs using GO terms, highlighting disease-associated genes. From the data, two genes were selected WSB2 (upregulated) and ATP6AP2 (downregulated). Total RNA was extracted from control and treatment groups of Gaddi dogs' PBMCs using the TRIzol method, and cDNA was synthesized with the Takara PrimeScript II Kit. RT-PCR validation was performed using the Bio-Rad CFX96 system and the Qiagen miScript SYBR Green PCR Kit. Primers were designed using NCBI Primer-BLAST and Primer3 Plus, and samples were analyzed in triplicate with melt curve analyses to ensure PCR product specificity. Relative gene expression was normalized to GAPDH and ACTB

and determined using the 2^-ΔΔCt method (Livak & Schmittgen, 2001).

RESULTS AND DISCUSSION

mRNA seq and data summary: The study explored the transcriptomic profile of Gaddi dog PBMCs stimulated with CpG ODN to understand their immune response to bacterial infections. RNA-Seq was conducted to analyze the differential gene expression and functional annotation upon exposure to TLR-9 ligands. The experiment generated 9.17 GB of raw data, with a mapping efficiency of 93.89% when aligned to the reference genome. Differential gene expression was inferred between samples using the R pheatmap package edger (Robinson *et al.* 2010) and was used for producing heatmap (Figure 1) and Volcano plot (Figure 2). A comprehensive DEG analysis for functional enrichment was conducted, focusing on DEGs with logFC ≥ 3 or ≤ -3 and a p-value < 0.05.

Identification of differentially expressed genes: A total of 1,072 DEGs (Table 1) were identified between the control and CpG-treated groups, with 506 (47.2%) significantly upregulated and 566 (52.79%) significantly downregulated. To better understand their functional implications, Gene Ontology (GO) and pathway enrichment analyses were performed separately on the upregulated and downregulated DEGs.

Gene ontology and pathway enrichment: The differential expression analysis revealed varying numbers of dysregulated transcripts. Pathway analysis and functional annotation were conducted using online tools such as DAVID, PANTHER, and STRING. For pathway enrichment data visualization and presentation, an online

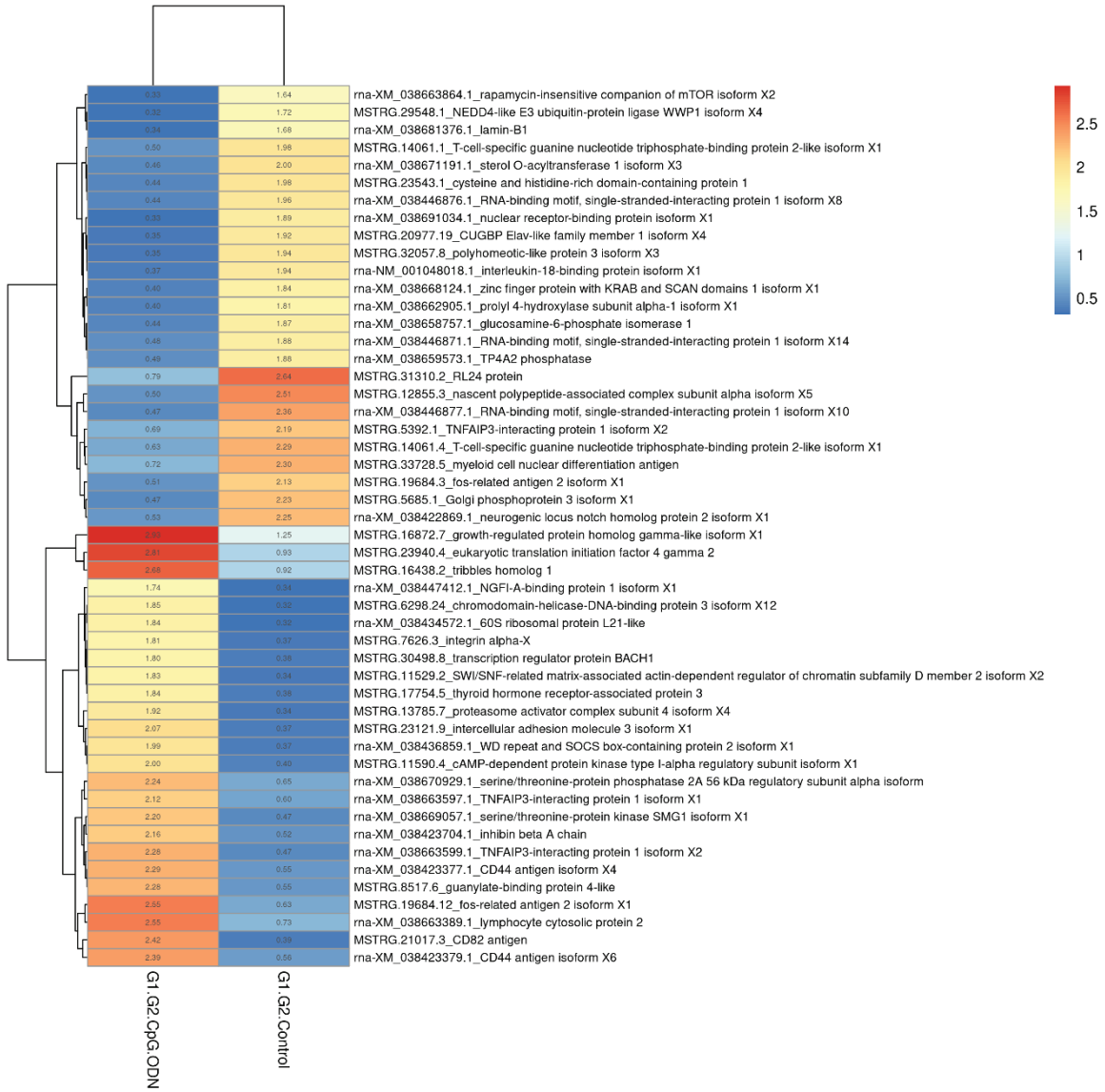

Fig. 1 Heatmap representing most significant genes expressed in Gaddi dog PBMC, Control, and CpG ODN treated groups. The samples were plotted using log10 of normalized read count values (CPM). shades of blue represent downregulated genes and shades of red represent highly expressed genes.

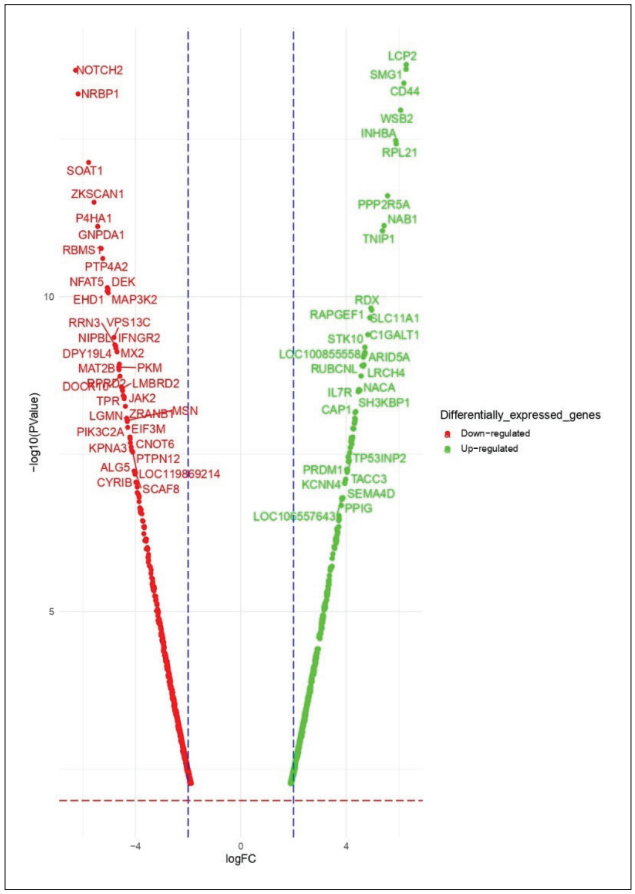

Fig. 2 Volcano Plot representing most significant genes expressed in Gaddi dog PBMC, Control, and CpG ODN treated group. The volcano plots display the log2 fold change (logFC) and -log10 p-value of differentially expressed genes (DEGs) with logFC ≥ 3 (highlighted green) or ≤ -3 (highlighted red) and p-value < 0.05.

platform Scientific and Research Plot tool was used (http://www.bioinformatics.com.cn/SRplot). The up-regulated DEG results have shown, autophagy, apoptosis NOD-like receptor, FoxO signaling pathway, chemokine signaling pathway, and NOD-like signaling pathway (Figure 3A). In contrast, the GO terms have shown, apoptosis and its regulation in leucocytes, indicating leucocytes apoptotic pathways, cell killing, and regulation of leucocyte apoptotic process (Figure 3B). TLR9 recognizes CpG motifs commonly found in bacterial and viral DNA, activating innate immune responses. This activation can drive various cellular processes, including apoptosis, particularly in immune cells like PBMCs. (Krogmann *et al.* 2016).

When PBMCs are stimulated with CpG ODNs, TLR9 is activated, leading to the production of pro-inflammatory cytokines such as TNF-α and IL-6. The cytokines, in turn, can contribute to the induction of apoptosis in specific immune cell subsets. This process is thought to be tightly regulated by dose and context. High doses of CpG ODNs, as shown in experimental models, can enhance pro-apoptotic pathways, potentially as a mechanism to control excessive inflammation and maintain immune homeostasis. (Xiang *et al.* 2015). Further, apoptosis following TLR9 stimulation has been linked to key pathways such as caspase activation, DNA fragmentation, and mitochondrial membrane potential loss, which are hallmarks of the apoptotic process. These effects suggest that TLR9 stimulation by CpG ODNs can be a double-edged sword, promoting immune responses while also leading to cell death in some contexts, which might serve as a regulatory feedback mechanism to prevent hyperactivation of immune responses. Research on CpG stimulation in PBMCs has highlighted the induction of a wide array of immune responses triggering the signaling cascades that activate pathways related to inflammation,

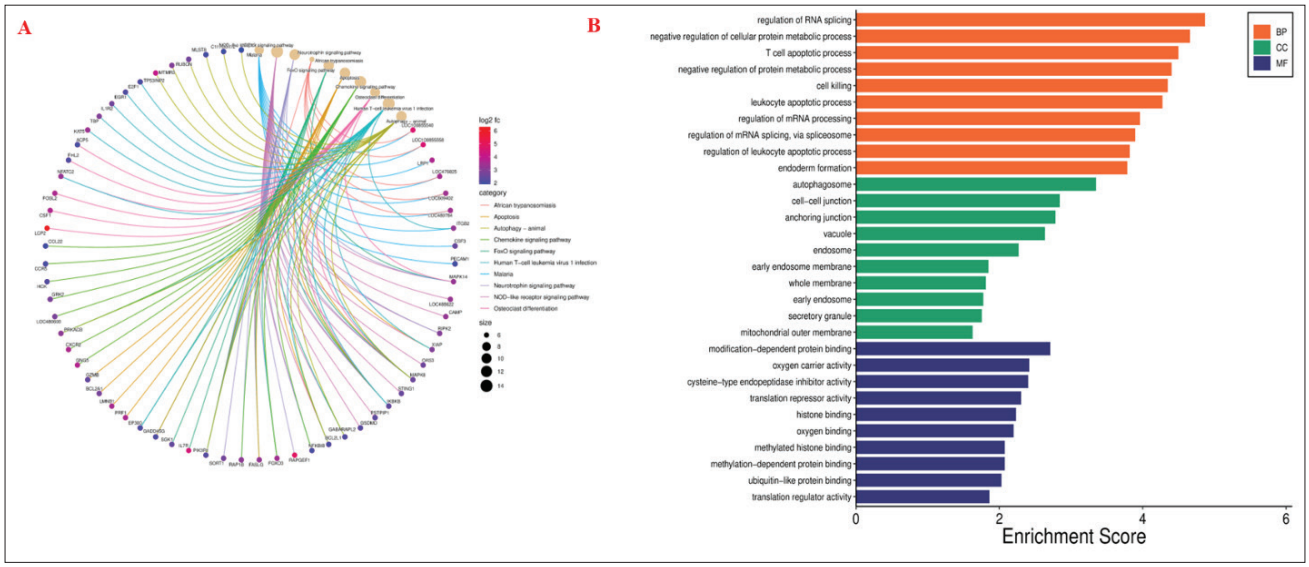

Fig. 3 Functional enrichment and GO analysis of Gaddi dog PBMC Upregulated Differentially expressed genes in CpG ODN treated groups. A. cneplot representing the enriched pathways. B. Gene Ontology terms showing the biological processes (BP), Cellular Components (CC), and Molecular Functions (MF). *Representation of functional enrichment was created from an online tool* (www.bioinformatics.com.cn/en)

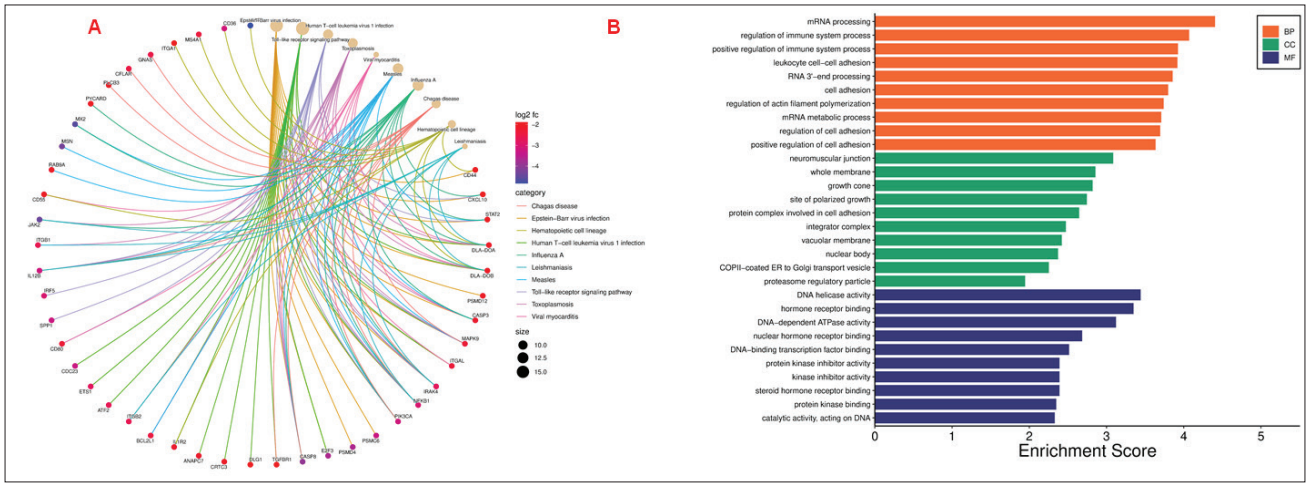

Fig. 4 Functional enrichment and GO analysis of Gaddi dog PBMC Downregulated Differentially expressed genes in CpG ODN treated groups. A. cneplot representing the enriched pathways. B. Gene Ontology terms showing the biological processes (BP), Cellular Components (CC), and Molecular Functions (MF). *Representation of functional enrichment was created from an online tool* (www.bioinformatics.com.cn/en)

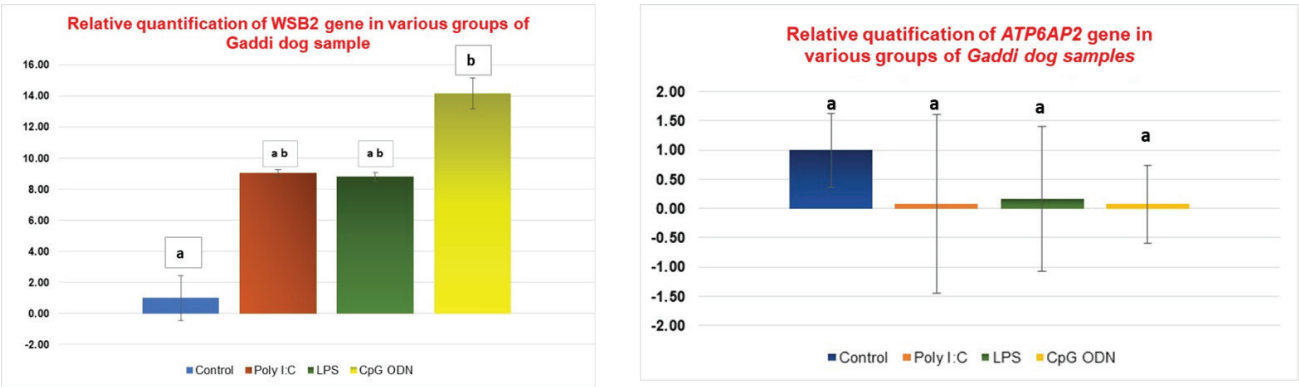

Fig. 5 Relative expression of mRNA Genes in *Gaddi* dog PBMCs in CpG ODN treated groups. WSB2 was significantly ($p<0.05$) upregulated, ATP6AP2 was downregulated ($p>0.05$). a, b: No common superscript between the levels of the effect indicates a significant difference at $p<0.05$

cytokine production, and apoptosis. Studies in humans and animals show that this stimulation can induce type I interferons (IFN-α, IFN-β), TNF-α, and pro-inflammatory cytokines like IL-6 and IL-8. The NOD-like receptor signaling pathway involves genes such as GSDMD, RIPK2, and BCL2L1, highlighting immune activation and inflammation (Almeida-da-Silva *et al.* 2023).

Moreover, the downregulated DEGs have shown the pathways related to various virus-related responses (Figure 4A). The GO terms have shown results in biological functions for mRNA processing, regulation of immune response, cell-cell adhesion, T-cell proliferation, intracellular protein transport, and regulation of stress response (Figure 4A), indicating the low transcription and translational processes and overcoming the response to CpG ODN ligand. The above observation indicates the strong immune response generated by the Gaddi dogs. These findings underscore the distinct transcriptomic landscape in Gaddi dogs reflecting their diverse immunogenetic adaptations and potential disease resistance mechanisms based on pathway analysis.

qRT- PCR validation of mRNA-seq: To verify the precision of our RNA-seq analyses, the upregulated DEGs and downregulated Gaddi dogs were selected for qPCR validation. WSB2 was significantly upregulated ($p<0.05$), impacting I21 receptor regulation and immune responses (Carneiro *et al.* 2021), while ATP6AP2 was downregulated but not significantly ($p>0.05$) confirming the accuracy and reliability of our transcriptomic data (Figure 5).

The present study presented the maiden report of the differential transcriptome profile of Gaddi dog PBMCs simulated with CpG ODN mimicking the Bacterial infection model. This study will support future functional studies and simulation experiments to identify immune related and disease-associated pathways.

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