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Gene expression profiles of the small intestine of village chickens from an *Ascaridia galli* infested environment

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ABSTRACT

Nematodes of the genus *Ascaridia* are known to infect many species of birds and result in fatal diseases. *A. galli* damages the intestinal mucosa of chickens leading to blood loss, secondary infection and occasionally the obstruction of small intestines due to high worm burden. This study investigated the gene expression profiles in chickens from two different provinces of South Africa naturally exposed to *A. galli* infestations and tested either positive or negative for the parasite. The study further investigated gene expression profiles of the *A. galli* infected duodenum, jejunum and ileum tissues of the small intestines. The *A. galli* positive intestines displayed hypertrophy of the intestinal villi with accumulation of inflammatory cells and necrosis of the crypts of Lieberkühn glands, lesions that were absent in the uninfected intestines. Total RNA isolated from small intestines of infected and non-infected intestines was sequenced using Illumina HiSeq technology to generate up to 23,856,130 reads. Between any two-way comparisons of the intestines, 277 and 190 transcripts were significantly expressed in Limpopo and KwaZulu-Natal (KZN) chickens, respectively. Gene ontology analysis of the differentially expressed genes (DEGs) revealed an enrichment of genes reported to function in the immune response, defense response, inflammatory response and cell signalling genes. *T cell* receptor signalling pathways and arachidonic acid metabolism pathways were among the most significantly impacted pathways. Overall, the study provided insights into adaptative mechanisms for chickens extensively raised in parasite infected environments.

1. Introduction

Village chickens are raised by smallholder farmers under low-input production systems (Mtileni et al., 2009), where they forage for their feed and as such are predisposed to parasites that coexist in the environment. Parasite infestation contributes to poor production and can cause mortality in severe cases (Daş et al., 2010; Gauly et al., 2005; Permin et al., 2006). Infection with *A. galli,* that has been reported in South African village chickens (Mukaratirwa and Khumalo, 2010; Mwale and Masika, 2011), contributes to economic losses due to poorer feed conversion, reduced weight gain and decreased egg production.

Genetic control strategies have been suggested as alternatives for disease and parasite control in chicken production systems (Lamont, 1998). Genetic resistance to infection is likely as a result of heritable genes or genomic loci that have a direct or indirect role in expression of molecules that appropriately regulate host immunity to control infection and limit pathology. Variants in these genes and/or genomic loci can be applied to breeding programs to enhance parasite resistance.

The normal habitat of the parasitic stages of *A. galli* is in the small intestine of poultry (Soulsby, 1982). The life cycle of *A. galli* is direct and involves a single host (Permin et al., 1997). Mature worms live in the lumen of the small intestine, whereas eggs containing infective stage larvae (L3) develop in the environment (Permin et al., 1997). The intestine, as a first barrier, is in contact with commensal and pathogenic microorganisms (Abreu, 2010). The intestinal epithelium is therefore a critical component of a communication network that is essential for transmitting signals generated in response to infection with pathogens to cells of the innate and acquired immune systems in the underlying intestinal mucosa. The response to changes allow the intestine to contribute to the defence against pathogens and to the control and regulation of the local immune response.

Identification of tissue-specific expression signatures has both theoretical and practical implications toward understanding host–parasite interactions. Gene expression influenced by infection can be measured

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on multiple tissues to give insight into the regulation of genes and pathways during infection. RNA sequencing (RNA-seq) is a high-throughput method developed for profiling transcriptomes that is cost-economic, time saving and can generate transcriptome data for non-model species (McIntyre et al., 2011; Wang et al., 2009). The approach allows gene expression profiling and in contrast to array-based techniques allows for the characterization of both known and unknown gene transcripts. Furthermore, data outputs are based on read counts instead of microarray hybridization intensity signals, which renders the technology feasible in studying variations in transcript sequences (Mortazavi et al., 2008). This study investigated the gene expression profiles of small intestines of South African village chickens with naturally acquired *A. galli* infestations. Furthermore, the three segments of the small intestine (duodenum, jejunum and ileum) were investigated independently in order to analyze variations of gene expression that could play a role in the site-specific functionalities of the gastro-intestinal tract. The end goal was to make inferences on genes, pathways and biological processes that play a role in resistance and tolerance to diseases and infections in village chickens exposed to gastrointestinal Nematode infestations with particular emphasis on *A. galli*.

2. Materials and methods

2.1. Chicken populations and intestinal tissue sample selection

A total of 144 non-descript adult village chickens were purchased from the villages in Limpopo (n = 99) and KwaZulu-Natal (KZN, n = 45) provinces. These chickens were reared under village chicken production system where no feed supplements or housing is provided and the environment is contaminated with parasites inclusive of *A. galli* (Malatji et al., 2016a, 2016b). The 144 chickens were euthanised by cervical disarticulation immediately after purchase. Of these 144 chickens, 40 were positive for *A. galli* infections, 18 of which were from Limpopo province and 22 from KZN province with worm burdens ranging from 1 to 150 worms per chicken. Intestinal tissue was sampled from chickens positive and negative for *A. galli* parasite from each province. The *A. galli* parasites that were recovered from Limpopo and KZN chickens indicated no significant genetic differentiation (Malatji et al., 2016a) indicating that a similar population of parasites was infecting chickens between the two provinces. From each of the selected chickens, 1.5–2 cm long jejunum, ileum and duodenum tissues were collected for histological examination. Each sample was opened along the mesenteric border and placed in tissue cassettes (32 mm, L x26 mm W, Themo Scientific) with wet filter papers to avoid curling and folding of the tissue.

2.2. Histology

A total of 86 and 97 *A. galli* parasites were recovered from the intestines of Limpopo and KZN positive chickens respectively. The intestinal tissue samples were fixed in 4% neutral buffered formaldehyde for 24 h and transferred to 70% ethanol. The samples were then processed conventionally, post-trimmed and embedded in paraffin. A 3_µm thick slide was made from the intestinal sample, mounted on conventional glass slides and stained with haematoxylin and eosin (HE) for overall evaluation. From every chicken two transverse sections of each intestinal sample were evaluated using light microscopy (Scale bar = 100 µm, x20).

2.3. RNA isolation from small intestine tissue

Three 60 mg tissue specimens were cut from each of the jejunum, ileum and the duodenum of the infected and non-infected chickens from KZN and Limpopo provinces. The three tissues from each section (jejunum, ileum and duodenum) of animal intestine were crushed using mortar and pestle into a homogenate from which total RNA was isolated for each section, using RNeasy Mini Kit (Qiagen), according to instructions of the manufacturer. RNA was eluted with 50 µl RNase-free water and stored at −80 °C. This was followed by a subsequent sample purification using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To remove contamination from genomic DNA, the RNA sample was DNase-treated using RNase-free DNase I (Qiagen). RNA concentration and quality were detected by measuring absorbance at 260 nm and A260/A280 ratio using NanoDrop (Thermo Scientific). The extent of RNA degradation was assessed by electrophoresis of 1 µg of RNA on a 1.8% agarose gel. The 12 RNA samples were stored in small aliquots at −80 °C until further use.

2.4. Sequencing using Illumina HiScan SBS technology

The TrueSeq RNA Sample Preparation kit (Illumina, San Diego, CA) was used for mRNA library preparations following the manufacturer's protocol. Agilent 2100 Bioanalyzer was used to monitor quality control on the samples. Sequencing was performed using Illumina HiSeq 2500 at the Agricultural Research Council-Biotechnology Platform, South Africa to generate 125bp paired-end data.

2.5. Quality control of sequence data

Quality control was first performed on the primary sequencing data produced by Illumina HiSeq 2500. Sequencing adapters were trimmed off from the reads. For each of the sequencing reads, low quality bases with quality score greater than 20 as determined by phred (Ewing and Green, 1998; Ewing et al., 1998) along 95% of the read length were also trimmed using CLC Bio workbench version 6.5 (CLC Bio, Aarhus, Denmark). Reads that were > 20 bp long on both sides of paired-end format were kept for further downstream analysis and were classified as high-quality reads.

2.6. Mapping of RNA-Seq reads to reference genome

Quality controlled reads were aligned to the *gallus.galgal 4.74* reference genome using TopHat v2.0.11 (Trapnell et al., 2009). TopHat uses Bowtie v2.1.0 (Langmead et al., 2009) to perform the alignment based on quality information accompanying each read, an initial alignment to the reference genome using default parameters. TopHat allowed a maximum of two mismatches when mapping reads to the reference and binary alignment map (BAM) files containing mapped and unmapped reads with a mapping report were produced.

2.7. Data preparation for transcript assembly and differential gene expression

Reads were assembled into transcripts using the default parameters of Cufflinks (Trapnell et al., 2012). The abundance of assembled transcripts was estimated and reported as fragments per kilobase of exon per million fragments mapped (FPKM), with confidence intervals estimated for each FPKM and filtering cut-off of 1.0. The DEGs ratios were tested for statistical significance using Bayesian statistics as described by Bullard et al. (2010). The significance scores were corrected for multiple testing using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). DEGs were defined as those with absolute value of log2 fold change higher than two and *P-value* was less than 0.05. DEGs for the whole small intestine and three regions of the small intestines were calculated using the ratio of *A. galli* infested versus normal FPKM values for every gene. Genes with expression level fold change between two conditions of more than 2, $p < 0.05$ and False discovery rate (FDR) < 0.05 were considered DEGs. Cufflinks constructed a minimum set of transcripts that best described the reads in the generated dataset. It also used the normalized RNA-Seq fragment counts to measure the relative abundances of transcripts. The unit of

measurement used was the FPKM. Confidence intervals for FPKM estimates were calculated using a Bayesian inference method (Jiang and Wong, 2009).

Cuffdiff was used to find significant changes in transcript expression, splicing, coding output and promoter use (Trapnell et al., 2012). It used the Cufflinks transcript quantification module to calculate transcript/gene expression levels and test them for significant changes. Cuffdiff used the reference transcripts as a gene transfer format (GTF) file and the three sequence alignment map (SAM) or binary version of SAM (BAM) files containing the fragment alignments of the three biological replicates of each sample of the three small intestine regions. To analyse whether genes were ubiquitously expressed among the targeted tissues, a venn diagram was plotted by using Venny, a freely accessible (http://bioinfogp.cnb.csic.es/tools/venny/index. Html) interactive tool for the comparison of lists. The multidimensional scaling plots (MDS), which measure the similarity of the samples and project it into two dimensions, were performed to measure the relationship between duodenum, jejunum and ileum using cummeRbund package in the R statistical environment (Trapnell et al., 2012). Principal component analysis (PCA) was also used for clustering and exploring the relationships between conditions. Graphs were generated by using cummeRbund and the ggplot2 package (Wickham, 2009). Visualisation of results was also undertaken using the cummeRbund package in R (Trapnell et al., 2012).

2.8. Functional annotation and pathway analysis

For each DEG, Gene Ontology (GO) annotation was done by using blast2go (Liu et al., 2011), which uses a blast algorithm to assign GO terms to sequences based on similarity. This was done to identify the key pathways and interactions that might be relevant for the response of intestinal tissues to *A. galli* infection and to annotate significant interactions with metabolic pathways. The DEGs between infected and non-infected intestinal tissues were classified into the categories of biological process, molecular function and cellular components. Bar graphs illustrating similarities and differences between GO terms were plotted using Web Gene Ontology Annotation Plot (WEGO), a web-based tool (Ye et al., 2006).

2.9. Validation of RNA-seq results with RT-qPCR

Real time quantitative PCR (RT-qPCR) was performed using QuantiTect SYBR Green kit (Qiagen Inc., Valencia, CA) to test the validity of specific gene transcription, RNA-seq data and variations in gene expression among individuals. The RNA used for qRT-PCR was prepared in the same way as the total RNA extraction and DNase I treatment described above. Five genes were selected based on their functions in the immune response as reported in other chicken studies and significance within the RNA-seq results (Pasick et al., 2017; Smith et al., 2015; Rychlik et al., 2014). Primer pairs were designed using Primer3 (Rozen and Skaletsky, 2000) and their primer sequences are listed in Table 1. A reference gene (*βeta-actin*) was used as endogenous control for normalization of the real-time PCR analysis. Reverse transcription (RT) of RNA was achieved using iScript Advanced first strand cDNA synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a total reaction volume of 20 μl following the manufacturer's instructions. Quantitative PCR was performed in triplicate for each cDNA sample on Roche LightCycler® 96 System (Roche Applied Science, US) using KAPA SYBR FAST Universal qPCR kit (KAPA Biosystems, SA) according to the manufacturer's instructions. Threshold lines were adjusted to intersect amplification lines in the linear portion of the amplification curve and cycles to threshold (Ct) were recorded. Standard curves for each gene and the reference gene were constructed using serial dilutions. The relative gene expression level was estimated using the comparative cycle threshold (CT) method (Livak and Schmittgen, 2001; Pfaffl, 2004). The method calculates the ratio of relative expression from the efficiency (E) and threshold cycle (C_t) values of an unknown sample versus a control sample.

3. Results

3.1. Histology

The three anatomical sections of the intestine of uninfected chickens had no evidence of histopathologic necrosis. There was no histological difference between the infected duodenum, jejunum and ileum of chickens from Limpopo and KZN provinces (Fig. 1, micrograph A, B and C). Intestines from *A. galli* positive duodenum, jejunum and ileum presented necrosis and hypertrophy of the intestinal villi with accumulation of inflammatory cells, and necrosis of the crypts of Lieberkühn glands and necrosis in the mucosa as shown in Fig. 1, micrograph D, E and F. Lymphocytes and macrophages were observed in the infected chickens. *A. galli* imposed similar impacts on the small intestine from Limpopo and KZN chickens.

3.2. RNA sequencing read mapping and differentially expressed genes

The number of reads (125 bp paired-end) generated ranged from 2.1million – 3.9 million per sample, over 80% of which were mapped to *gallus.galgal 4.74* reference genome.

A total of 55,014 genes were identified from the whole organism in this study. Of these, 277 (0.50%) and 190 (0.35%) genes were differentially regulated in response to *A. galli* infection in the samples representing Limpopo and KZN provinces respectively. Comparisons were made between negative and positive chickens from Limpopo (LIM_N/P comparison) and KZN (KZN_N/P comparison). Among the DEGs in the LIM_N/P comparison, 124 (44.77%) were up regulated and 153 (55.23%) down regulated in *A. galli* infected chickens. A total of 34 DEGs were shared between KZN_N/P and LIM_N/P comparisons (Fig. 2). In KZN_N/P comparison, 80 (42.11%) were up regulated and 110 (57.89%) down regulated. There were 156 and 243 DEGs that were only present in the KZN_N/P comparison, or LIM_N/P comparison, respectively. Some of the genes identified in LIM_N/P and KZN_N/P comparisons are listed in Table 2 and Table 3 respectively. Transcriptome analysis of the three regions of the small intestine from Limpopo chickens identified 76, 99 and 78 DEGs in the duodenum,

Table 1
Primer sequences of selected differentially expressed genes used for RT-qPCR. *βeta-actin* was used as a reference gene for normalization of the RT-qPCR analysis.

Primer name	Forward	Reverse
FABP1	GGGAAGAGTGTGAGATGGA	CCCCTTCAAGTTAGCAACCA
APOA4	GAACTAACACGCGCGCTAATT	TTCAGGTCTTCATGCGCTTCT
DRD4	TGGGCTCCAGACTGTCTCTT	GATCTTGGCTCGCTTCTGAC
BLB2	CCGCAGCGTTCTTCTTCTAC	CCGGTTGTAGATTTGCCTGT
MR1	CACGTGTTTGGAGTTTGTGG	TCATCCCCAGGA AATAATGC
*βeta-Actin**	ACGTCTCACTGGATTTCGAGCAGG	TGCATCCTGTCAGCAATGCCAG

Gene used as reference is marked *, Primers were designed using Primer3 program.

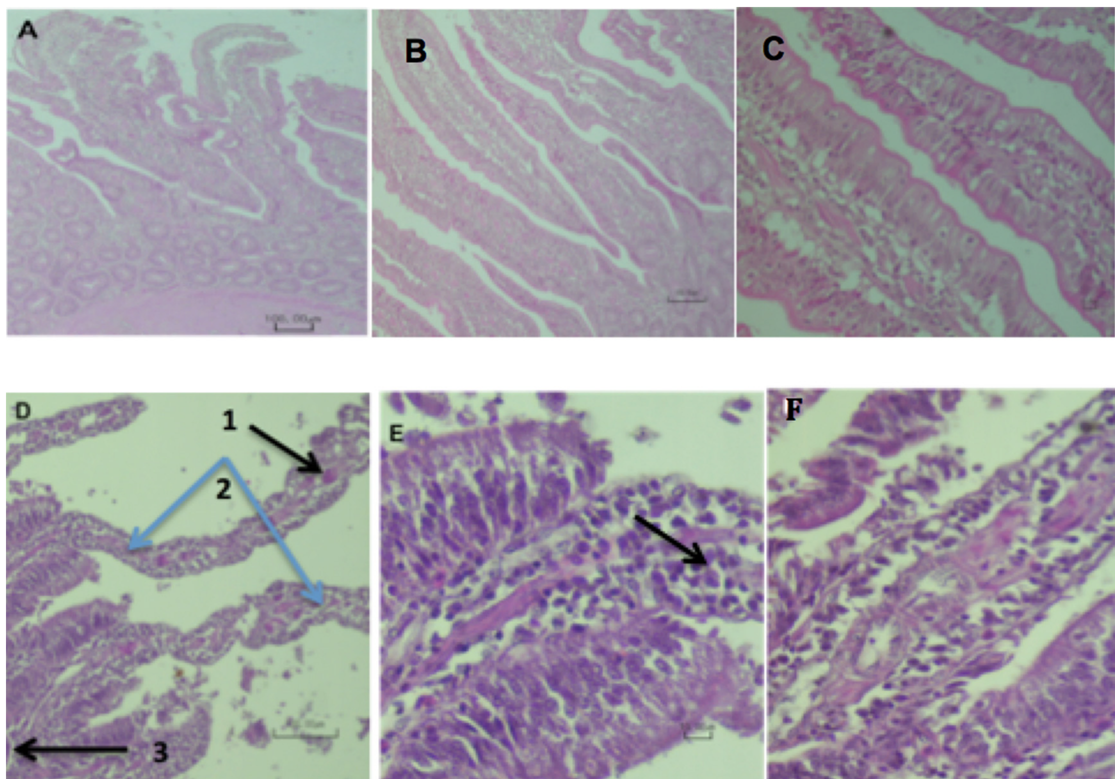

Fig. 1. Photomicrograph of a section of a normal (A) and *A. galli* infested (B) Small intestine. Small intestine infested by *A. galli* worms (C). Necrosis of the crypt of Lieberkühn gland and villi were observed in infected small intestine.

jejunum and ileum sections respectively. In tissues from KZN, 33, 15 and 18 DEGs were identified in the duodenum, jejunum and ileum sections of the intestine respectively (Table 4).

A total of 31 genes were co-expressed in all the sections of the small intestines collected from Limpopo and 10 genes where common in the three sections collected from KZN. Three (3) genes were differentially expressed in all the analysed samples (Fig. 3). Only 16 genes were exclusively expressed in duodenum while 47 and 25 genes could be detected only in the jejunum and ileum of chickens from Limpopo. In chickens from KZN, 15 genes were exclusively expressed in the duodenum while 50 and 137 genes could be detected only in the jejunum and ileum respectively (Fig. 3). Some annotated genes showed similar

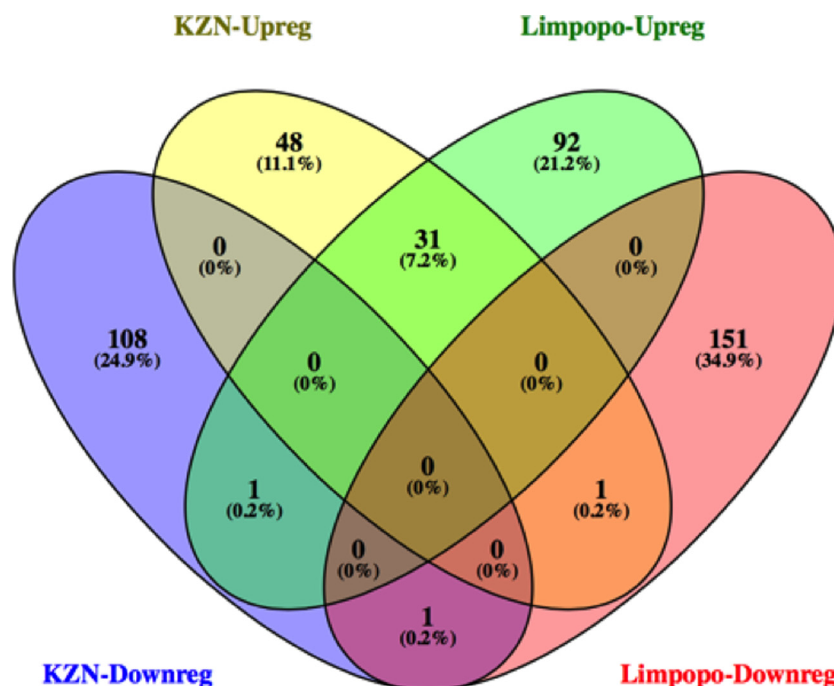

Fig. 2. Overlapping differentially expressed genes (up or down-regulated). Common genes were observed in comparisons between infected and non-infected small intestines of chickens from KwaZulu-Natal and Limpopo provinces.

Table 2
Differential gene expression between Limpopo village chicken intestines that were negative and positive for *A. galli* parasites.

Limpopo up-regulated	Seq. Description	RPKM_N	RPKM_P	log2(fold_change)	p_value
		5.74292	337.971	5.87897	0.00055
	senescence-associated protein	1944.51	178020.0	6.51649	5,00E-05
GOLGA4	low quality protein: golgin subfamily a member 4	1944.51	178020.0	6.51649	0.0057
	hypothetical protein CP8484711_2381, partial	4.46432	125.568	4.81388	0.00125
SYNPO2	synaptopodin- partial	1.16224	21.6793	4.22134	0.0041
	phosphatidylinositol -bisphosphate 5-phosphatase a-like	2.37946	39.1956	4.04198	0.0041
TLR7	tlr7	0.636696	8.0061	4.25142	0.0026
HPS5	hermansky-pudlak syndrome 5 protein	10.2695	124.333	3.59778	0.001
	PREDICTED: spidroin-1-like, neurofilament medium polypeptide isoform x1	86.7866	800.611	3.20556	5,00E-05
IL21R	interleukin-21 receptor	1.05924	9.72641	3.19887	0.0033
	envelope partial, pol partial	28.3597	218.111	2.94315	0.0001
IL20RA	Interleukin-20 receptor subunit alpha	5.68072	41.4622	2.86765	4.2073
	transitional endoplasmic reticulum partial, low quality protein: syncytin-1-like	33.9977	164.989	2.27886	0.0001
HSP30C	heat shock protein 30c-like	1.40715	9.57623	2.76668	0.006
HSPB7	heat shock protein beta-7	3.05223	10.6764	1.80649	0.0014
Limpopo down regulated					
	hypothetical protein A306_09416	56.8579	2.14685	−4.72707	5,00E-05
LOC100857191	c-c motif chemokine 21	420.328	16.2807	−4.69028	0.002
CXCR4	c-x-c chemokine receptor type partial	35.5861	2.67279	−3.7349	5,00E-05
	cd209 antigen-like protein a	48.9896	3.73815	−3.71208	0.0005
	class i histocompatibility f10 alpha chain-like isoform x1	368.999	29.9511	−3.62294	5,00E-05
	cyclic amp-dependent transcription factor atf-3 isoform x2	26.7937	2.29904	−3.54279	5,00E-05
HSP105	heat shock protein 105 kda isoform x2	47.0572	4.5253	−3.37833	5,00E-05
	tyrosine-protein phosphatase non-receptor type 7	111.45	14.4115	−2.95111	5,00E-05
SLAMF1	signalling lymphocytic activation molecule	88.0741	16.2089	−2.44193	0.0039
	t-cell surface glycoprotein cd3 epsilon chain	94.6376	20.5752	−2.20151	0.0015

P- value of < 0.05 is significant.

patterns of expression between different sections of the intestine, which included c-c motif chemokine ligand 21 that was down-regulated in duodenum and ileum of positive chickens from Limpopo. Furthermore, a similar pattern of expression was also observed with tyrosine-protein kinase jack3.

3.3. Multidimensional scaling plots

Investigation of the MDS plot produced an apparent structure within the data that is consistent with tissue segments. The plot revealed tissue dissimilarity of gene expression patterns between tissues by clearly separating duodenum, jejunum and ileum segments. This was observed in both Limpopo and KZN provinces (Fig. 4a(I) and 4b(I). In agreement with the MDS plot, a PCA plot based on principal components 1 and 2 resulted in a distinct separation based on tissue segments (Fig. 4a[II] and 4b[II])

3.4. Gene ontology (GO) analyses

In general, fewer significantly enriched GO terms were found in the KZN-N/P (Additional file 1) compared to the Limpopo-N/P (Additional file 2) comparison. GO terms at level 2 were used as representatives (Fig. 5). Of these GO terms, biological processes such as immune response (GO:0006955), response to stimulus (GO:0050896), immune system process (GO:0002376) and signalling (GO:0023052) were enriched. Binding GO such as dopamine receptor activity (GO:0001591)

Table 3
Differential gene expression between KZN village chicken intestines that were negative and positive for *A. galli* parasites.

Gene Name	Seq. Description	RPKM_N	RPKM_P	log2(fold_change)	p_value
KZN up-regulated					
ATF7IP	activating transcription factor 7-interacting protein 1 isoform x1	3.2018	179.746	5.81093	5,00E-05
	collagen alpha-1 chain-like	98.7552	4324.89	5.45266	0.0002
LOC419333	formin-f- partial	1.35691	24.1984	4.15651	0.0033
HPS5	hermansky-pudlak syndrome 5 protein	13.1854	293.446	4.47608	5,00E-05
CSF2RB	cytokine receptor common subunit beta- partial	0.471599	7.52509	3.99608	5,00E-05
TRAF3IP3	traf3-interacting jnk-activating modulator isoform x1	0.759631	8.20497	3.43313	0.00335
	Cytochrome P450 2h1-like isoform x1	4.29065	35.8769	3.06378	5,00E-05
	rna-binding protein 25 isoform x2	5.68072	41.4622	2.86765	5,00E-05
FABP5	fatty acid-binding epidermal	12.8834	92.782	2.84834	0.0027
KZN Down-regulated					
DAK	bifunctional atp-dependent dihydroxyacetone kinase fad-amp lyase	2148.25	82.2961	−4.7062	5,00E-05
APOA	apolipoprotein a-iv	784.167	33.1607	−4.56361	0.0015
	cgmp-dependent protein kinase 1-like	925.85	47.8782	−4.27334	0.0029
	sperm-associated antigen 5	88.3557	8.22701	−3.42488	0.00465
DPEP1	dipeptidase 1	171.901	17.6539	−3.28352	5,00E-05
	maltase- partial	311.361	32.8363	−3.24522	0.0033
	solute carrier family 26 member 6	130.194	16.6938	−2.96328	5,00E-05
	testican-2	304.962	39.9486	−2.93241	0.00015
HSP70	Heat shock protein 70	76.4445	9.57394	−2.99723	0.00015
	interleukin- partial	175.379	17.7204	−3.30699	5,00E-05

P- value of < 0.05 is significant.

Table 4
Gene expression statistics for comparisons between negative and *A. galli* positive sections of the small intestine of village chickens from KZN and Limpopo provinces.

Province	Limpopo			KZN		
Section of the intestine	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Significantly up & down regulated genes	76	99	78	33	102	158
Significantly up-regulated genes $p < 0.05$	37	58	34	15	41	16
Significantly down-regulated genes $p < 0.05$	39	41	44	18	61	142
Non-significant genes $p > 0.05$	34976	34953	34974	35019	34950	34894
Number of Sequences	123	64	78	32	79	123
Sequences with blast hits e-value $\leq 1E-10$	91	41	40	20	51	91
Sequences without blast hits	32	23	38	12	28	32

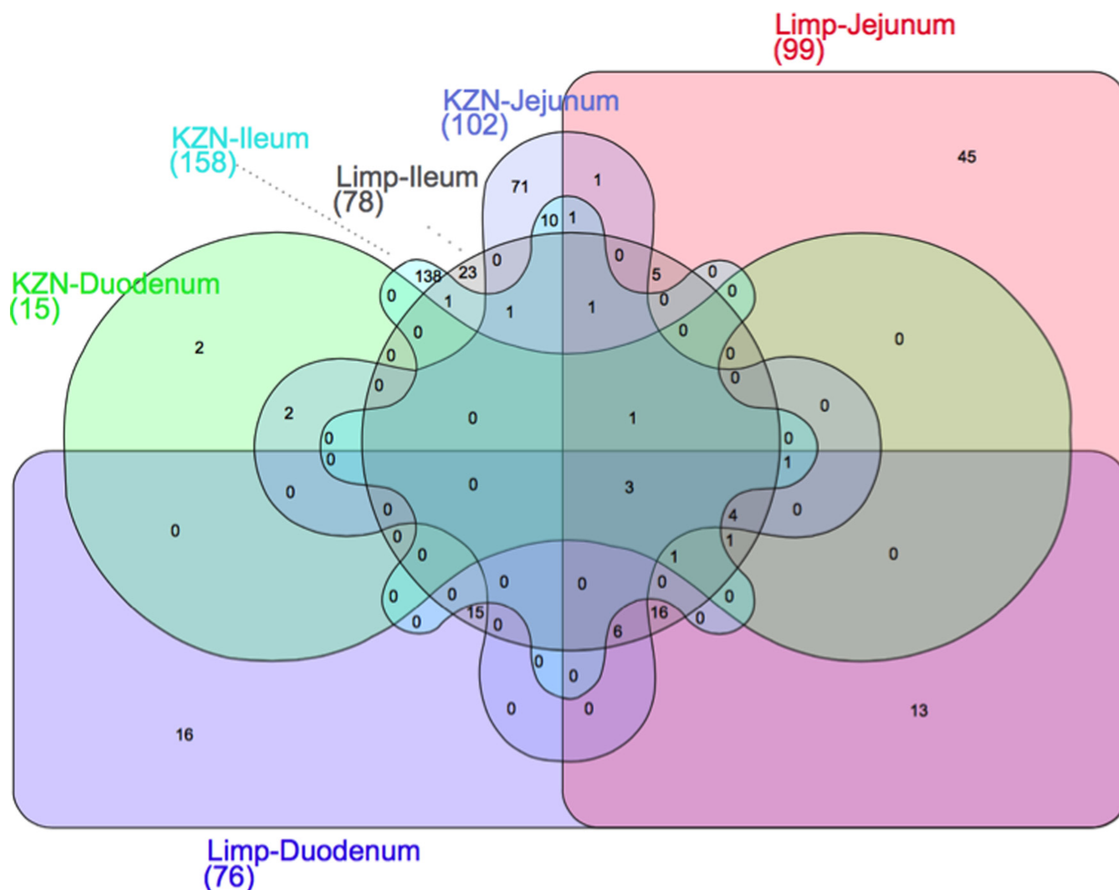

Fig. 3. Overlapping differentially expressed genes (up or down-regulated). Common genes were observed along sections of the smal intestines of chickens from KwaZulu-Natal and Limpopo provinces.

and ATP binding (GO:0005524) along with cellular processes such as cellular response to stimulus (GO:0051716) and cellular response to stress (GO:0033554) had the highest percentage of transcripts involved. The most predominant biological functions were common amongst the different anatomical segments of the small intestine. The first five predominant biological processes observed for tissue samples from Limpopo provinces were cellular process, biological regulation, metabolic processes, pigmentation and response to stimulus. The GO profiles were similar between specimens from Limpopo and KZN provinces with the exception of biological regulation that was not in the top three of the biological process term in KZN. Immune response terms that included immune system process, response to stimuli and cell killing were also observed in the presence of *A. galli* infection.

3.5. Biological pathways associated with chicken's response to A. galli infections

To gain an understanding into pathways involved in the village chicken's reaction to *A. galli* infection, gene annotation was conducted using enzyme code and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The top 5 KEGG pathways associated with the most abundant differentially up-regulated (Fig. 6) genes in infected and non-infected chickens from Limpopo included T cell receptor signalling pathway, Drug metabolism - cytochrome P450, Metabolism of xenobiotics by cytochrome P450, histidine metabolism and arachidonic acid metabolism. The impacted T cell receptor signalling pathway involved tyrosine-protein kinase btk, tyrosine-protein kinase csk and tyrosine-protein kinase jak3 genes that play a major role in the immune response. In KZN chickens, pathways impacted included arachidonic acid (Fig. 7), methane metabolism, glycerolipid metabolism, purine metabolism, glutathione metabolism and cyanoamino acid metabolism. Arachidonic acid and purine metabolism were observed to have been impacted in tissues from both provinces. However, genes involved in these pathways were different for the two provinces.

Pathway analysis was also undertaken using all the up and down regulated genes from the three sections of the small intestine (Fig. 6).

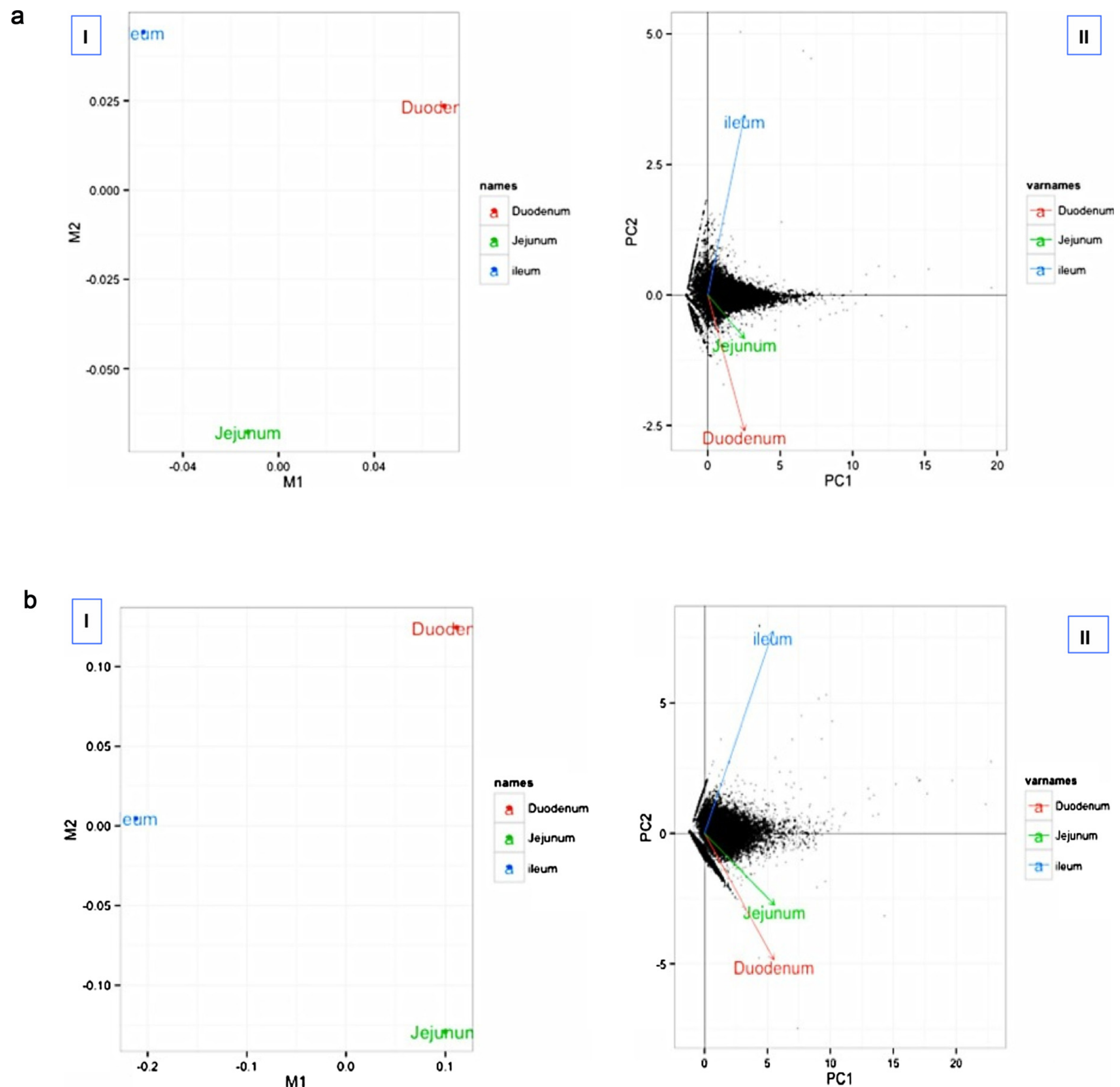

Fig. 4. (a) Multidimensional scaling analysis and principal component analysis. MDS and PCA analysis using expression of all genes of small intestine tissues from Limpopo provinces. (b) Multidimensional scaling analysis and principal component analysis. MDS and PCA analysis using expression of all genes of small intestine tissues from KZN provinces.

Genes up-regulated in the ileum of KZN chickens impacted the most pathways. In Limpopo, the jejunum had the most impacted pathways. Other pathways were impacted in only one or two sections of the intestine. In Limpopo, T cell receptor pathway was impacted in the duodenum and ileum and not the jejunum. Most of the pathways had only one gene involved except for purine metabolism that had four genes involved, followed by Tryptophan metabolism and Pyrimidine metabolism with two genes each. The Linoleic acid metabolism, Arachidonic acid metabolism, Metabolism of xenobiotics by cytochrome P450, Drug metabolism - cytochrome P450 and Purine metabolism pathways were commonly impacted in both provinces. The common pathways were however not impacted in the same sections of the intestine.

3.6. Validation of RNA-seq results using RT-qPCR

RT-qPCR was performed to validate the RNA-seq results with reference to *βeta-actin* control gene. Genes were selected based on their functions in immune response and significance within the RNA-seq findings. Of all the five genes tested, *MR1* gene was not differentially expressed in the RT-qPCR results (Table 5). However, the other genes were consistent between RNA-seq and RT-qPCR results.

4. Discussion

Nematodes of the genus *Ascaridia* are known to infect many species of birds causing fatal diseases. The small intestine is a principal target organ of *A. galli* infection (Katakam et al., 2010; Luna-Olivares et al., 2012; Taylor et al., 2007). Previous study (Fatihu et al., 1990; Luna-Olivares et al., 2012) showed that most of the helminth parasites were restricted to the small intestine, particularly the duodenum where there are optimum concentrations of saline and glucose. The histopathology of the three anatomical sections of the infected small intestines of chickens from Limpopo and KZN showed signs of damage. The histology findings imply different roles of the different sections in the life cycle of *A. galli* similar to reports from previous studies (Idi, 2004; Kaufmann, 1996; Soulsby, 1982; Luna-Olivares et al., 2012). The exact time of infection could not be established as the chickens were naturally infected with the *A. galli* parasites. However, adult mature worms were collected from the small intestines suggesting that the infection was at

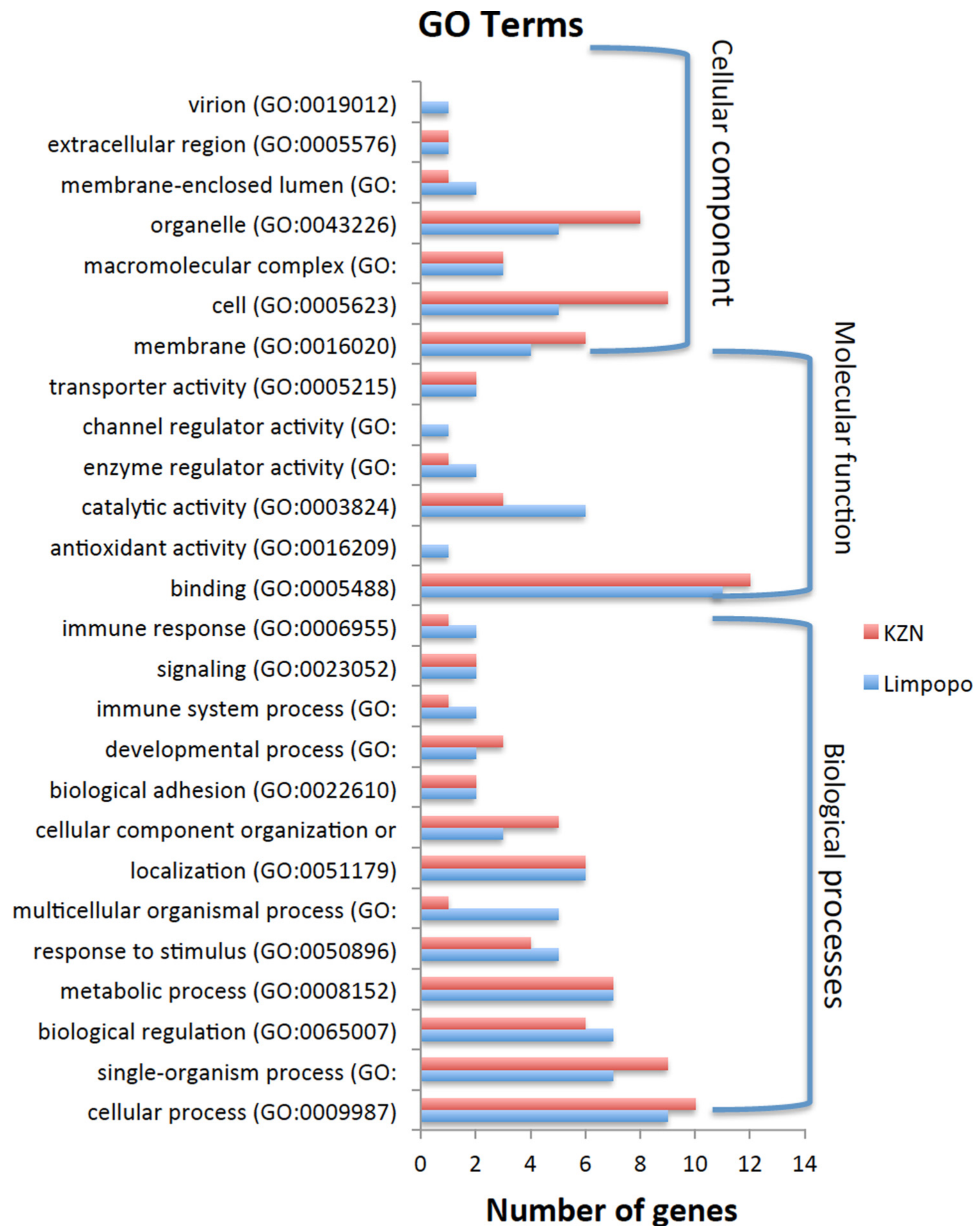

Fig. 5. Enriched gene ontology terms. Ontology categories resulting from interactions between chicken and *A. galli* infection from Limpopo and KZN.

the late-stage.

An average of 3.97 million paired-end reads per sample were generated, providing sufficient sequence coverage for transcriptome profiling (Sultan et al., 2008). An average of 84.82% of these reads were mapped to the reference chicken genome (*gallus.galgal4.74*). This suggested that the majority of the reads were of high quality and within RNA-Seq quality standards (Mortazavi et al., 2008) for downstream analysis.

The study reported altered transcriptomes as evidenced by DEGs between the *A galli* infected and non-infected intestines. The small intestines of chickens from Limpopo and KZN chickens that were infected by *A. galli* differed in the quantitative composition of the differentially expressed genes implying differences in the genetic mechanisms between the two chicken populations. More genes and pathways were involved in chickens from Limpopo versus those from KZN province.

Complementary to whole organism analysis, this study provided a finer resolution of the molecular processes happening in the different sections of the small intestine. Danos (2013) observed the strong positive correlation between libraries prepared from pooled RNA samples (pooled RNA from 3 biological replicates) and those from libraries prepared separately from biological replicates. As such and due to financial and computational resource constraints, we decided to use the "pooled" RNA approach to reduce the cost of sequencing. The results that were validated with RT-qPCR method revealed that the different sections of the small intestine differed in the quantitative composition of the genes expressed in response to *A. galli* infection. The duodenum, jejunum and ileum displayed distinct transcriptome characteristics supporting the hypothesis that different regions of the intestine are impacted differently during nematode invasion. It was not surprising that the ileum, which is the lower small intestine, known to be associated with increased immune surveillance had the largest or second largest number of expressed genes in chickens from KZN and Limpopo provinces respectively. In a study by Freeman et al (2012), the largest cluster of genes associated with the immune system were observed to be expressed two- to three-fold higher in the ileum relative to the other regions.

Several genes involved in the immune response were observed to be differentially expressed in this study. The DEG analysis indicated that

Fig. 6. KEGG pathways enriched in differentially expressed genes in Limp_N/P and KZN_N/P. The pathways associated with chicken's response to *A. galli* infections.

the *MHC class II m alpha chain precursor* was up regulated whilst *MHC class II partial* was down regulated in chickens from Limpopo. *Class I histocompatibility f10 alpha chain-like isoform x1&2* were also down regulated in Limpopo. The MHC (Major Histocompatibility Complex) is a group of genes present in all vertebrates that plays a major role in immune response mechanisms through molecules known to regulate different cellular components of the immune system including the T cells, B cells, and antigen-presenting cells (Lamont, 1998). The chicken MHC (B complex) has been implicated with conferring either resistance or susceptibility to a variety of viral, parasitic, and bacterial diseases (Lamont, 1998).

Pro-inflammatory cytokines genes were also observed to be differentially expressed in in the study population. The expression of pro-inflammatory cytokine genes are known to be activated by toll-like receptors (*TLRs*) in order to directly control parasite replication (Gazzinelli and Denkers, 2006). *Interleukin-20 receptor subunit alpha and*

Fig. 7. Arachidonic acid metabolism pathhway. Genes involved in the pathway are shown in pink. Genes involved in the pathway are shown in pink. Arachidonic acid was observed to have impacted tissues from Limpopo and KZN provinces.

Table 5
Comparison of gene expression levels (fold changes) between RNA-seq and qRT PCR. *βeta-actin* was used as a reference gene for normalization of the RT-qPCR analysis.

Comparison	Method	*APOA4*	*MR1*	*FAB1*	*DRD*	*BLB2*
Duodenum N/P[a]	RNA-seq			2.85		
	RT-qPCR			0.57		
Jejunum N/P	RNA-seq	3.11			2.41	
	RT-qPCR	2.04			0.46	
Ileum N/P	RNA-seq		1.58	2.61		1.43
	RT-qPCR		0.78[b]	1.79		0.73

[a] N = Negative; P = Positive.
[b] Represents gene that was not significantly differentially expressed in RT-qPCR results.

interleukin-21 receptor were up-regulated in Limpopo chickens whereas *interleukin- partial* was down-regulated in KZN. *Interleukin-22* is a member of the interleukin 10 related cytokine family (Zenewicz et al., 2008). The *interleukin-22 receptor* is highly expressed within tissues, such as epithelial cells of the gastrointestinal tract.

Different families of pattern recognition receptors (*PRRs)* have been identified (Mair et al., 2004) including among other receptors, *TLRs* (O'Neill, 2006). *TLRs* play a central role in the initiation of immune responses against a number of pathogens (Ingham et al., 2008). They recognize various *pathogen-associated molecular patterns (PAMPs)* from parasites (Kawai and Akira, 2001). *TLR7* is commonly involved in pathogen recognition, and was up-regulated (4.2 fold increase) in infected chickens from Limpopo (Limp5). In a separate study (Ingham et al., 2008), *TLR7* transcript level changes were observed in sheep resistant to *H. contortus*. Previous studies on the identification of avian *TLRs* were primarily dependent on bioinformatics (in silico) approaches (Temperley et al., 2008; Yilmaz et al., 2005). Limited information is therefore available for avian *TLRs'* functions and mechanisms of regulation. This study provides unique information important in understanding the involvement of avian *TLRs* in the immune response to nematode infections.

Cytochrome P450s (*CYPs*) are a large superfamily of enzymes found in almost all living organisms. In this study, *Cytochrome P450* 2h*1-like isoform x1* was down regulated in infected KZN chickens and not differentially expressed in the chickens from Limpopo region. In a study on malabsorption syndrome, which is a model for intestinal disturbances in young chickens (van Hemert et al., 2004), *Cytochrome P450* and *apolipoprotein B* showed differences in expression among lines. *Apolipoprotein a-iv* was down regulated in chickens from KZN. Previous studies reported down regulation of *apolipoprotein B* and *cytochrome P450* in intestinal epithelium in response to pro inflammatory cytokines (Besnard et al., 2002; Darmawi et al., 2013). Based on such evidence, the down-regulation of *apolipoprotein* and *cytochrome P450* in this study, might be a response to intrusion in the small intestine.

The *CXC chemokine receptor type partial* and *CXC chemokine 21* were down regulated in the small intestines of *A. galli* infected chicken from Limpopo province. *CXC chemokines IL-8* and *K60* were up regulated in the jejunum of *Salmonella serovar Typhimurium* infected chicken (Withanage et al., 2004). A *Th2* polarised cytokine response was reported in the jejunum and spleen of *A. galli*-infected chickens (Kaiser, 2007; Pleidrup et al., 2014). An increased expression of the Th2 signature cytokine IL-13 in the spleen of *A. galli*-infected chickens was also observed in this study. *CXC* activates natural killer cells and is thought to play a role in the temporal development of innate and adaptive immunity (Neville et al., 1997). As suggested by results from this study, *CXC* seem to have a major role in the defense of chickens from *A. galli.* This gene family also was also proven to trigger necrosis (Gasser and Newton, 2000), which is consistent with the histology results from this study.

Heat shock protein 30c-like and *heat shock protein beta-7* were up regulated while *heat shock protein 105 partial* was down regulated in Limpopo chickens. *Heat shock protein 30c-like* belongs to the small heat shock protein 20 family and is involved in stress response. *Heat shock protein 70*, which can also play a significant role in heat stress, underwent significant down-regulation in infected chicken from KZN. This stress protein was also expressed in other studies (Kim et al., 2014).

Notably, this study revealed several genes that were specific to different segments of the intestine and are important in immune response mechanisms. MDS plots highlighted the separation of the three targeted sections of the intestine into three distinct groups, results of which were consistent with the PCA analysis. Taken together, both the MDS and PCA suggest significant biological dissimilarities between segments, which may be a result of the tissue's degree of specialization. Results from this study are consistent with reports by Mach et al (2014) who investigated gene expression patterns along the proximal-distal axis of the porcine small intestine (duodenum, jejunum and ileum) and observed an obvious structure along tissue segments.

The analysis of enriched GO terms enabled the discovery of significant gene categories that could have been overlooked by evaluating individual genes. The enriched GO terms could aid in interpreting the dominant functions controlled by DEGs. The higher number of identified GO terms was positively correlated to the number of DEGs in all comparisons. This trend was observed in both up and down regulated genes. The enriched biological processes such as response to stimulus, immune system process and signalling are all involved in immune response and could be associated with the resistance or tolerance of chickens to *A. galli* parasites.

The biological pathway analysis gave insight into the molecular mechanisms underlying protective immunity and host resistance to *A. galli* parasitic infection. Arachidonic acid metabolism was one of the pathways significantly impacted in *A. galli* infected chickens from KZN. Arachidonic acid is capable of modifying or regulating one or more immune functions and may serve as a potent inhibitor for Type 1 helper T cell (Th1) response (Freeman et al., 2012). It is one of the crucial polyunsaturated fatty acids associated with membrane phospholipids which when liberated from the plasma membrane, can be oxidized to a variety of eicosanoids. Eicosanoids act in signalling molecules and stimulating a variety of responses in their target cells including an immune response (Peters-Golden et al., 2005). Worm killing activities of arachidonic acid have previously been demonstrated in a study conducted in mice where a single oral dose of arachidonic acid led to a significant reduction of total worm burden of Schistosoma.

Another pathway mostly impacted was the T cell receptor signalling pathway. The ability of Nematodes to destabilize the TLR signalling pathway has been reported previously (Kane et al., 2004). Our results suggest a role of T-cell receptor signalling pathway in invoking effective host immune responses and in the development of host resistance.

5. Conclusion

The study used RNA-seq data to successfully identify and analyse DEGs from small intestine collected from village chickens that were naturally infected with *A. galli*. There were noteworthy differences in the mechanisms of host resistant to *A. galli* infestation between chickens from different provinces, which could be due to variance in genetic profiles of the two populations. The data presented in the study could find application in development of genomic markers for use in selection and breeding of chickens against natural infections with pathogens such as *A. galli*. Understanding the molecular mechanisms that contribute to protective immunity, immune suppression, pathology, and host resistance will have a significant impact on alternative disease/Pathogen control strategies.

Availability of data and materials

The datasets supporting the conclusions of this article are available

in the NCBI Short Read Archive (SRA) repository, SRP070940.txt.

Competing interests

There authors declare that they have no competing interests.

Authors' contributions

DPM performed lab work, analysed data and drafted the manuscript. FCM was the project leader and engaged in the structure of the manuscript. EvM participated in the coordination and preparation of the manuscript. All authors were responsible for experimental and project design.

Ethical standards

Ethical approval for the study was obtained from the Animal Ethics Committee from the Agricultural Research Council-Animal Production Institute(APIEC13/004) and University of Pretoria (EC090-13).

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References

Abreu, M.T., 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat. Rev. Immunol. 10, 131–144.
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B. Stat. Methodol. 57, 289–300.
Besnard, P., Niot, I., Poirier, H., Clement, L., Bernard, A., 2002. New insights into the fatty acid-binding protein (FABP) family in the small intestine. Mol. Cell Biochem. 239, 139–147.
Danos, C., 2013. Methods for comprehensive transcriptome analysis using nextgeneration sequencing and application in hypertrophic cardiomyopathy. Harvard University.
Darmawi, D., Balqis, U., Hambal, M., Tiuria, R., Frengki, F., Priosoeryanto, B., 2013. Mucosal mast cells response in the jejunum of Ascaridia galli-infected laying hens. Media Peternak 36, 113–119.
Daş, G., Kaufmann, F., Abel, H., Gauly, M., 2010. Effect of extra dietary lysine in Ascaridia galli-infected grower layers. Vet. Parasitol. 170, 238–243.
Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8, 186–194.
Ewing, B., Hillier, L., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces usingPhred. I. Accuracy assessment. Genome Res. 8, 175–185.
Fatihu, M., Ogbogu, V., Njoku, C., Saror, D., 1990. Comparative studies of gastrointestinal helminths of poultry in Zaria, Nigeria. Revue d'élevage et de médecine vétérinaire des pays tropicaux 44, 175–177.
Freeman, T., Ivens, A., Baillie, J., Beraldi, D., Barnett, M., 2012. A gene expression atlas of the domestic pig. BMC Biol. 10, 90.
Gasser, R., Newton, S., 2000. Genomic and genetic research on bursate nematodes: significance, implications and prospects. Int. J. Parasitol. 30, 509–534.
Gauly, M., Homann, T., Erhardt, G., 2005. Age-related differences of Ascaridia galli egg output and worm burden in chickens following a single dose infection. Vet. Parasitol. 128, 141–148.
Gazzinelli, R., Denkers, E., 2006. Protozoan encounters with toll-like receptor signalling pathways: implications for host parasitism. Nat. Rev. Immunol. 6, 895–906.
Idi, A., 2004. Effect of selected micronutrients and diets on the establishment and pathogenicity of *Ascaridia galli* in chickens. The Royal Veterinary and Agricultural University, Copenhagen, Denmark.
Ingham, A., Reverter, A., Windon, R., Hunt, P., Menzies, M., 2008. Gastrointestinal nematode challenge induces some conserved gene expression changes in the gut mucosa of genetically resistant sheep. Int. J. Parasitol. 38, 431–442.
Jiang, H., Wong, W.H., 2009. Statistical inferences for isoform expression in RNA-Seq. Bioinformatics 25, 1026–1032.
Kaiser, P., 2007. The avian immune genome – a glass half-full or half-empty? Cytogenet. Genome Res. 117, 221–230.
Katakam, K.K., Nejsum, P., Kyvsgaard, N.C., J⊘ rgensen, C.B., Thamsborg, S.M., 2010. Molecular and parasitological tools for the study of Ascaridia galli population dynamics in chickens. Avian Pathol. 39, 81–85.
Kaufmann, J., 1996. Parasitic infection of domestic animals: a diagnostic manual. Birkhauser Verlag, Basel. 357–358.
Kawai, T., Akira, S., 2001. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34, 637–650.
Kim, D., Yoo, W., Lee, M., Yang, H., Kim, Y., Cho, S., Lee, W., Ju, J., 2014. Transcriptome sequencing and analysis of the zoonotic parasite Spirometra erinacei spargana (plerocercoids). Parasit. Vectors 7, 368.
Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25.
Liu, S., Lin, L., Jiang, P., Wang, D., Xing, Y., 2011. A comparison of RNA-Seq and high-density exon array for detecting differential gene expression between closely related species. Nucleic Acids Res. 39, 578–588.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402–408.
Luna-Olivares, L.A., Ferdushy, T., Kyvsgaard, N.C., Nejsum, P., Thamsborg, S.M., Roepstorff, A., Iburg, T.M., 2012. Localization of Ascaridia galli larvae in the jejunum of chickens 3 days post infection. Vet. parasitol. 185, 186–193.
Mach, N., Berri, M., Esquerré, D., Chevaleyre, C., Lemonnier, G., Billon, Y., Lepage, P., Oswald, I.P., Doré, J., Rogel-Gaillard, C., 2014. Extensive expression differences along porcine small intestine evidenced by transcriptome sequencing. PloS One 9, e88515.
Mair, K., Sedlak, C., Kaser, T., Pasternak, A., Levast, B., Gerner, W., 2004. The porcine innate immune system: an update. Dev. Comp. Immunol. 45, 321–343.
Malatji, D., Tsotetsi, A., van Marle-Köster, E., Muchadeyi, F., 2016a. Population genetic structure of Ascaridia galli of extensively raised chickens of South Africa. Vet. Parasitol. 216, 89–92.
Malatji, D., Tsotetsi, A., van Marle-Köster, E., Muchadeyi, F., 2016b. Village chicken production systems and their influence on gastro-intestinal parasites infections: South African case study. Onderstepoort J. Vet. Res. 83, a968.
McIntyre, L., Lopiano, K., Morse, A., Amin, V., Oberg, A., Young, L., Nuzhdin, S., 2011. RNA-seq: technical variability and sampling. BMC Genomics 12, 293.
Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B., 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, 621–628.
Mtileni, B., Muchadeyi, F., Maiwashe, A., Phitsane, P., Halimani, T., Chimonyo, M., Dzama, K., 2009. Characterisation of production systems for indigenous chicken genetic resources of South Africa. Appl. Anim. Husbandry Rural Dev. 2, 18–22.
Mukaratirwa, S., Khumalo, M., 2010. Prevalence of helminth parasites in free-range chickens from selected rural communities in KwaZulu-Natal province of South Africa. J. S. Afr. Vet. Assoc. 81, 97–101.
Mwale, M., Masika, P.J., 2011. Point prevalence study of gastro-intestinal parasites in village chickens of Centane district, South Africa. Afr. J. Agric. Res. 6, 2033–2038.
Neville, L.F., Mathiak, G., Bagasra, O., 1997. The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the CXC chemokine superfamily. Cytokine Growth Factor Rev. 8, 207–219.
O'Neill, L., 2006. How Toll-like receptors signal: what we know and what we don't know. Curr. Opin. Immunol. 18, 3–9.
Pasick, J., Diederich, S., Berhane, Y., Embury-Hyatt1, C., Wanhong, X., 2017. Imbalance between innate antiviral and pro-inflammatory immune responses may contribute to different outcomes involving low-and highly pathogenic avian influenza H5N3 infections in chickens. J. Gen. Virol. 98, 1245–1258.
Permin, A., Christensen, J.P., Bisgaard, M., 2006. Consequences of concurrent Ascardia galli and Escherichia coli infections in chickens. Acta Vet. Scand. 47, 43–54.
Permin, A., Pearman, M., Nansen, P., Bisgaard, M., Frandsen, F., 1997. On investigation in different media for embryonation of Ascaridia galli eggs. Helminthologia 34, 75–79.
Peters-Golden, M., Canetti, C., Mancuso, P., Coffey, M.J., 2005. Leukotrienes: under-appreciated mediators of innate immune responses. J. Immunol. 174, 589–594.
Pfaffl, M.W., 2004. Quantification strategies in real-time PCR. AZ of quantitative PCR 1, 89–113.
Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biology programmers. Methods Mol. Biol. 132, 365–386.
Rychlik, I., Elsheimer-Matulova, M., Kyrova, K., 2014. Gene expression in the chicken caecum in response to infections with non-typhoid Salmonella. Vet Res. 45, 119–132. https://doi.org/10.1186/s13567-014-0119-2.
Smith, J., Smith, N., Yu, L., Paton, I.R., Gutowska, M.W., Forrest, H.L., Danner, A.F., Seiler, J.P., Digard, P., Webster, R.G., et al., 2015. A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. BMC Genomics 16 (1), 574. https://doi.org/10.1186/s12864-015-1778-8.
Soulsby, E.J.L., 1982. Helminths, arthropods and protozoa of domesticated animals. Bailliere Tindall.
Sultan, M., Schulz, M.H., Richard, H., Magen, A., Klingenhoff, A., Scherf, M., Seifert, M., Borodina, T., Soldatov, A., Parkhomchuk, D., 2008. A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. Science 321, 956–960.
Taylor, M., Coop, R., Wall, R., 2007. Parasites of poultry and gamebird. In: Veterinary Parasitology, 3rd edn. Blackwell Publishing.
Temperley, N.D., Berlin, S., Paton, I.R., Griffin, D.K., Burt, D.W., 2008. Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. BMC Genomics 9, 62.
Trapnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7, 562–578.
van Hemert, S., Hoekman, A., Smits, M., Rebel, J., 2004. Differences in intestinal gene expression profiles in broiler lines varying in susceptibility to malabsorption syndrome. Poult. Sci. 83, 1675–1682.

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 10, 57–63.
Wickham, H., 2009. ggplot2: elegant graphics for data analysis. Springer Science & Business Media.
Withanage, G., Kaiser, P., Wigley, P., Powers, C., Mastroeni, P., Brooks, H., Barrow, P., Smith, A., Maskell, D., McConnell, I., 2004. Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with Salmonella enterica serovar typhimurium. Infect. Immun. 72, 2152–2159.
Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., Wang, J., Li, S., Li, R., Bolund, L., 2006. WEGO: a web tool for plotting GO annotations. Nucleic Acids Res. 34, W293–W297.
Yilmaz, A., Shen, S., Adelson, D.L., Xavier, S., Zhu, J.J., 2005. Identification and sequence analysis of chicken Toll-like receptors. Immunogenetics 56, 743–753.
Zenewicz, L., Yancopoulos, G., Valenzuela, D., Murphy, A., Stevens, S., Flavell, R., 2008. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity 29, 947–957.