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Improved resistance to *Eimeria acervulina* infection in chickens due to dietary supplementation with garlic metabolites

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Abstract

The effects of a compound including the secondary metabolites of garlic, propyl thiosulphinate (PTS) and propyl thiosulphinate oxide (PTSO), on the *in vitro* and *in vivo* parameters of chicken gut immunity during experimental *Eimeria acervulina* infection were evaluated. In *in vitro* assays, the compound comprised of PTSO (67 %) and PTS (33 %) dose-dependently killed invasive *E. acervulina* sporozoites and stimulated higher spleen cell proliferation. Broiler chickens continuously fed from hatch with PTSO/PTS compound-supplemented diet and orally challenged with live *E. acervulina* oocysts had increased body weight gain, decreased faecal oocyst excretion and greater *E. acervulina* profilin antibody responses, compared with chickens fed a non-supplemented diet. Differential gene expression by microarray hybridisation identified 1227 transcripts whose levels were significantly altered in the intestinal lymphocytes of PTSO/PTS-fed birds compared with non-supplemented controls (552 up-regulated, 675 down-regulated). Biological pathway analysis identified the altered transcripts as belonging to the categories 'Disease and Disorder' and 'Physiological System Development and Function'. In the former category, the most significant function identified was 'Inflammatory Response', while the most significant function in the latter category was 'Cardiovascular System Development and Function'. This new information documents the immunologic and genomic changes that occur in chickens following PTSO/PTS dietary supplementation, which are relevant to protective immunity during avian coccidiosis.

Key words: Garlic: Propyl thiosulphinate oxide: Propyl thiosulphinate: *Eimeria acervulina*: Chickens: Microarray techniques

Garlic (*Allium sativum*) is a medicinal plant that has historically been used for the treatment and prevention of some diseases and has recently been shown to have antimicrobial, antitumour, antioxidant and immunostimulatory properties[1–4]. The pharmacological effects of garlic on cardiovascular disease (CVD) are mediated through the alteration of cholesterol and triglyceride (TAG) levels in the bloodstream[5]. The medicinal effects of garlic are derived from its flavonoid and organosulphur components. Studies in mammalian systems have indicated that these active pharmacological components act on multiple intracellular signalling pathways. In chickens, garlic compounds have also been shown to lower cholesterol and exert immunomodulating and antimicrobial activities[6–9]. However, compared with human studies, there is relatively limited information on the use of phytonutrients in general, and garlic in particular, in poultry veterinary medicine.

In addition to its effects on the cardiovascular system, garlic has been suggested for the treatment of parasitoses and other intestinal diseases[10]. For example, crude extracts of garlic reduced or eliminated *Hymenolepis*, *Aspiculuris*, *Histomonas* and *Eimeria* parasites in animal models of infection[11–15]. Of these parasites, coccidia protozoa comprise a subclass of single-celled eukaryotic micro-organisms belonging to the phylum Apicomplexa and class Conoidasida. Coccidia of the genus *Eimeria* infect the intestinal epithelia of chickens, turkeys and some mammalian hosts.

The intestinal intraepithelial lymphocytes (IEL), the primary immune effector cells of the gut-associated lymphoid tissues, recognise and destroy pathogens that breach the intestinal epithelium[16]. Chicken intestinal IEL are composed of two phenotypically and functionally distinct subpopulations, natural-killer (NK) cells and T-lymphocytes[17]. In our previous studies, we have shown that IEL are the major effectors against coccidia parasites, *Eimeria*[18,19]. In chickens, disruption of the gut

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hank's balanced salt solution; IEL, intraepithelial lymphocytes; NK, natural killer; PTS, propyl thiosulphinate; PTSO, propyl thiosulphinate oxide; RPMI, Roswell Park Memorial Institute; TLR, Toll-like receptor; *TNFSF13B*, TNF (ligand) superfamily member 13b.

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epithelium during avian coccidiosis severely restricts the feed conversion efficiency during commercial poultry production. While in-feed anticoccidial drugs and antibiotic growth promoters can be used to mitigate some of the effects of avian coccidiosis, the emergence of drug-resistant parasites and legislative restrictions on the use of in-feed antibiotic growth promoters are driving the development of alternative disease control strategies for poultry production[20,21]. Because garlic has been shown to exert anticoccidial effects in mouse and rabbit infection models[14], in the present study we evaluated the effects of a compound including garlic-derived secondary metabolites, propyl thiosulphinate (PTS) and propyl thiosulphinate oxide (PTSO), on intestinal immunity to *E. acervulina*.

Materials and methods

Parasite cytotoxicity assay

Freshly sporulated *E. acervulina* oocysts were disrupted with 0·5 mm glass beads for 5–7 s and the freed sporocysts were incubated for 45 min at 41°C in PBS containing 0·014 M-taurodeoxycholic acid (Sigma) and 0·25 % trypsin (Sigma) to release infective sporozoites. Sporozoites were separated from debris by filtration, resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen), and isolated by centrifugation at 2100 ***g*** for 10 min at 4°C. Isolated sporozoites ($1{\cdot}0 \times 10^6$/ml) were incubated with PBS (negative control), 10 μg/ml of Garlicon40® or 5·0 μg/ml of chicken purified recombinant NK-lysin (positive control)[22] for 2 or 4 h at 4°C, and viability was measured by trypan blue exclusion by counting a minimum of 100 sporozoites.

Garlicon40®, a product containing 40 % of a compound made of 67 % of propyl thiosulphinate oxide (PTSO) and 33 % of PTS, was provided by Pancosma S.A. The biosynthesis of PTS is made from propiin (S-propyl-L-cysteine sulphoxide), an amino acid derived from L-cysteine found in *Allium* species. The first step of the biosynthesis (of thiosulphinates) is the formation of the sulphenic acid associated (in this case propyl-1-sulphenic acid; $CH_3CH_2CH_2SOH$) plus pyruvate plus ammonia. However, these compounds (sulphenic acids) are highly reactive, so they immediately produce thiosulphinates by a condensation reaction. In the last step, heating PTS induces its dismutation in PTSO and propyldisulphide. Propyldisulphide can be oxidised and transformed to PTSO and the oxidation of PTS to PTSO is never totally completed. Therefore, the two molecules are generally present in commercial samples. Garlicon40® was notated as PTSO/PTS in the present study.

Spleen lymphocyte proliferation

All experiments were approved by the Agricultural Research Service Institutional Animal Care and Use Committee. For the purpose of the experiment, 3-week-old Ross/Ross broiler chickens (Longenecker's Hatchery) were euthanised by cervical dislocation, and the spleens were removed and placed in Petri dishes with 10 ml of Hank's balanced salt solution (HBSS) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma). Cell suspensions were prepared by gently flushing through a cell strainer and lymphocytes were purified by density gradient centrifugation through Histopaque-1077 (Sigma). The cells were adjusted to $1{\cdot}0 \times 10^7$ cells/ml in RPMI-1640 medium without phenol red (Sigma) and supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Then, 100 μl/well of the sample were added to ninety-six-well flat-bottomed plates containing 100 μl/well of PTSO/PTS (10, 5, 2·5, 1·25, 0·6 or 0·3 μg/ml) from Pancosma S.A., 5 μg/ml of concanavalin A (Sigma) as a positive control, or medium alone as a negative control. The cells were incubated at 41°C in a humidified incubator (Forma) with 5 % CO_2 for 48 h, and the cell numbers were measured using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) (Dojindo Molecular Technologies) at 450 nm using a microplate spectrophotometer (Bio-Rad).

Experimental animals and diets

Broiler chickens (Ross/Ross, Longenecker's Hatchery) were randomly housed in Petersime starter brooder units and fed, from hatch, a standard diet alone (control) or a diet supplemented with 10·0 parts per million of PTSO/PTS. The composition of the standard diet was prepared as recommended by the National Research Council[23]. The PTSO/PTS dose was chosen based on preliminary dose–response experiments.

Eimeria acervulina infection of chickens

At 10 d post-hatch, the chickens were transferred to large hanging cages (two birds/cage) and were orally infected with $1{\cdot}0 \times 10^4$ sporulated oocysts of *E. acervulina*. The remaining chickens were housed in neighbouring cages, but were uninfected. Body weights (twenty birds/group) were measured at 0 and 10 d post-infection. Faecal samples (twenty birds/group) were collected between 6 and 9 d post-infection, and oocyst numbers were determined using a McMaster chamber (HK Inc.) according to the formula[24]: total oocysts/bird = oocyst count × dilution factor × (faecal sample volume/counting chamber volume)/2.

Anti-profilin serum antibody assay

Peripheral blood (four birds/group) was collected from uninfected and infected chickens at 10 d post-infection, and sera were prepared by centrifugation and analysed for anti-profilin antibody levels by ELISA. For this, ninety-six-well plates were coated overnight with 10 μg/well of *E. acervulina* purified recombinant profilin protein as described[25]. The plates were washed with PBS containing 0·05 % Tween and blocked with PBS containing 1·0 % bovine serum albumin (Sigma). Diluted sera at 1:50 were added (100 μl/well), incubated with agitation for 1 h at room temperature and washed with PBS containing 0·05 % Tween. Bound antibody was detected with horseradish peroxidase-conjugated rabbit anti-chicken IgG (Sigma) and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma) by measuring the optical density at 450 nm. All samples were analysed in quadruplicate.

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Microarray hybridisation

Chicken intestinal IEL were isolated from the non-infected chickens at 14 d post-hatch as described[26] and gene expression analysis was performed by microarray hybridisation. The intestinal jejunum was removed, cut longitudinally and washed three times with ice-cold HBSS. Then, the samples were incubated in HBSS containing 0·5 mM-EDTA and 5 % fetal calf serum for 20 min at 37°C with constant swirling. Cells released into the supernatant were passed through nylon wool (Robbins Scientific), and washed twice with HBSS containing 5 % fetal calf serum. The IEL were purified by Percoll density gradient centrifugation and washed three times with HBSS containing 5 % fetal calf serum.

In preliminary experiments, we determined that the percentage of CD45-positive IEL ranges from 45 to 70 % depending upon the genetics, age and infection status of chickens used (data not shown). Total RNA (six birds/group) was isolated using Trizol (Invitrogen) and pooled in two samples with equal amount from three birds each. RNA were amplified using the Two-Color Quick Amp Labeling Kit (Agilent Technologies) with cyanine 3 (Cy3)- or Cy5-labelled CTP. The RNA probes from the control and treatment groups labelled with two different colours were hybridised with one Chicken Gene Expression Microarray (Agilent Technologies) containing 43 803 elements. Then, two biological replicates were conducted with alternation of Cy3- and Cy5-labelled RNA to prevent data distortion from sample labelling[27]. Microarray images were scanned, and data extraction and analysis were performed using Feature Extraction software version 10.7.3.1 (Agilent Technologies).

Microarray data analysis

GeneSpring GX10 software (Silicon Genetics) was used to qualify and normalise image analysis data and to determine the fold changes in gene expression. Average signal intensities were corrected for background signals and normalised by the locally weighted regression and smoothing scatter plots method. Flag information was applied to strain the spots with 100 % valid values from each sample and an asymptotic *t* test analysis was performed to analyse the significance between PTSO/PTS-fed and non-supplemented control groups. To generate signal ratios, signal channel values from PTSO/PTS-fed birds were divided by values from negative controls. Modulated elements were defined as RNA with $>2{\cdot}0$-fold increased or decreased levels with $P<0{\cdot}05$, as determined by the Student's *t* test. The differentially expressed genes were filtered using the Volcano Plot method[28] built by the *t* test. All microarray information and data were deposited in the Gene Expression Omnibus database (series record no. GSE36302).

Quantitative RT-PCR

Gene expression changes observed by microarray analysis were confirmed by quantitative RT-PCR as described[29]. Equivalent amounts of the same RNA samples used for microarray hybridisations were reverse-transcribed using the StrataScript First Strand Synthesis System (Stratagene). Oligonucleotide primers are listed in Table 1. Amplification

Table 1. Primers used for quantitative RT-PCR

Symbol	Gene name	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplification efficiency*	GenBank accession no.
ADIPOR2	Adiponectin receptor 2	GTCACCTCAAGAAGACGAAG	GTTGTCCTTTAGCCAGTCAG	1·9776	NM_001007854.1
ASPH	Asp β-hydroxylase	CGTCGGTTACCTCTTGATAG	TGTATGCCTCTTTGTCTCCT	1·9821	XM_419224.2
IL-8	IL-8	CCTGCCTCCTACATTCATAC	AGAAAGACCTGCAGAGAGTG	1·6714	NM_205018.1
TNFSF13B	TNF (ligand) superfamily, member 13b	GTAACGGACTGTGGTGAACT	GGATTTCAGACCTCTTTCCT	1·5783	NM_204327.2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA	2·2253	K01458

* Calculated by the Q-gene program. Efficiency = $10^{(-1/\text{regression slope})}$.

and detection were carried out using the Mx3000P system and Brilliant SYBR Green quantitative RT-PCR master mix (SABioscience, Stratagene). Standard curves were generated using $\log_{10}$ diluted standard RNA and the levels of individual transcripts were normalised to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the Q-gene program[30]. For the calculation of fold changes between treatment groups, the cycle threshold (C_t) value of the target gene was normalised to GAPDH and calibrated to the relevant control value. Each analysis was performed in triplicate.

Bioinformatics data analysis

Differentially expressed genes between the PTSO/PTS-fed and control chickens were analysed using Ingenuity Pathway Analysis software (IPA) (Ingenuity Systems). Each identifier was mapped to its corresponding gene in IPA (Ingenuity Systems). Both 2·0-fold increased and 2·0-fold decreased identifiers were defined as value parameters for the analysis. These genes, termed 'focus genes', were superimposed onto the global molecular networks contained within IPA (Ingenuity Systems). Functional gene analysis was performed to identify the biological functions and canonical pathways of genes from the mapped data sets. The Fisher's exact test was used to calculate *P* values to assess the probability of each biological function and pathway assigned to that data set. The pathways of focus genes were algorithmically generated based on their connectivity.

Statistical analysis

Data from parasite cytotoxicity assays, body weight gains, oocyst shedding, spleen lymphoproliferation and profilin antibody levels were expressed as the mean and standard deviation values. Comparisons of the mean values were performed by one-way ANOVA followed by Duncan's multiple-range test or Student's *t* test using SPSS software (SPSS 15.0 for Windows); differences between groups were considered statistically significant at $P<0·05$.

Results

Effect of dietary propyl thiosulphinate oxide/propyl thiosulphinate on in vitro sporozoite viability and spleen cell proliferation

Treatment of freshly prepared *E. acervulina* sporozoites with 10 μg/ml of PTSO/PTS for 2 or 4 h significantly decreased parasite viability by 18 and 71 %, respectively (Fig. 1A). The reduced sporozoite viability was comparable with that produced by 5·0 μg/ml of chicken purified recombinant NK lysin, previously shown to be cytotoxic for *E. acervulina* and *E. maxima* parasites[22].

PTSO/PTS significantly increased splenocyte proliferation at 2·5 μg/ml or higher concentrations tested compared with the medium control at $P<0·001$ (Fig. 1B).

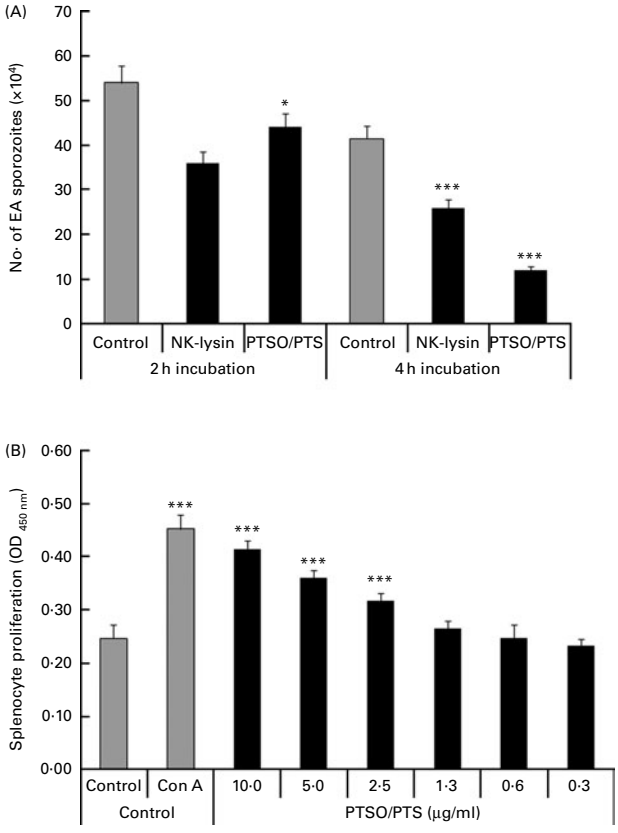

Fig. 1. Effect of propyl thiosulphinate oxide/propyl thiosulphinate (PTSO/PTS) *in vitro*. (A) *Eimeria acervulina* (EA) sporozoites ($1·0 \times 10^6$/ml) were incubated with PBS (control), 10 μg/ml of PTSO/PTS or 5·0 μg/ml of chicken recombinant natural killer (NK) lysin, for 2 or 4 h at 4°C, and viability was measured by trypan blue exclusion by counting a minimum of 100 sporozoites. (B) Spleen cells were treated with the indicated concentrations of PTSO/PTS, concanavalin A (Con A; 5 μg/ml) or medium (control) for 48 h and viable cell numbers were measured using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium. Values are means, with standard deviations represented by vertical bars (*n* 3). Mean values were significantly different from those of PTSO/PTS-treated with control groups according to the Student's *t* test: * $P<0·05$; *** $P<0·001$. OD, optical density.

Effect of dietary propyl thiosulphinate oxide/propyl thiosulphinate on body weight gain and faecal oocyst excretion following experimental Eimeria acervulina infection

Dietary supplementation with PTSO/PTS significantly increased body weight gain in *E. acervulina*-infected chickens compared with infected birds given a non-supplemented control diet (Fig. 2A). In addition, chickens fed a diet supplemented with PTSO/PTS showed decreased faecal oocyst excretion compared with non-supplemented controls (Fig. 2B).

Effect of dietary propyl thiosulphinate oxide/propyl thiosulphinate on serum anti-profilin antibody levels

Serum antibody levels against *E. acervulina* profilin were measured as a parameter of humoral immunity in PTSO/PTS-supplemented and non-supplemented chickens. The profilin antibody levels were higher in *Eimeria*-infected chickens than

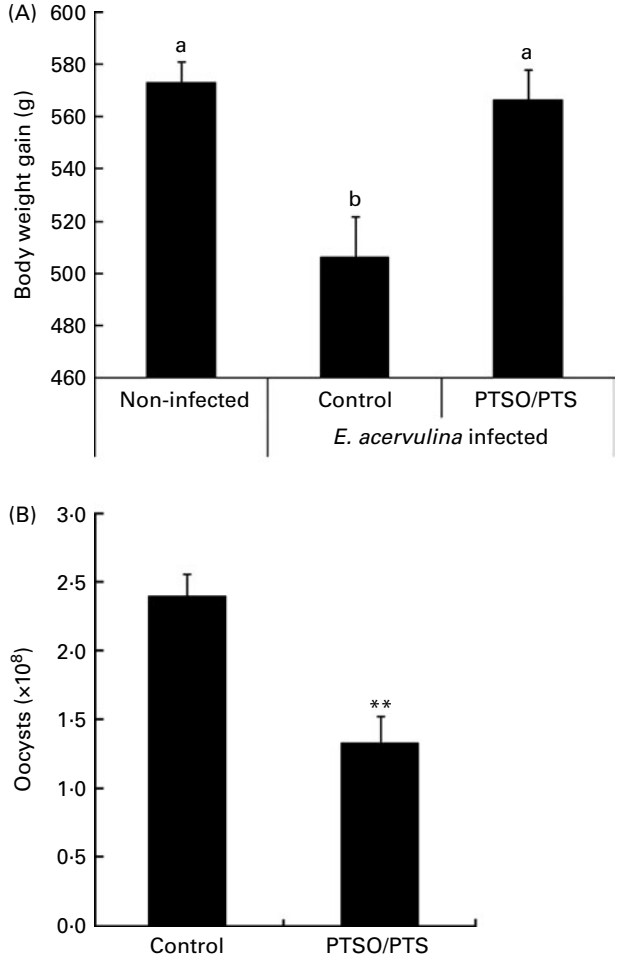

Fig. 2. Effect of dietary propyl thiosulphinate oxide/propyl thiosulphinate (PTSO/PTS) on body weight gain and faecal oocyst excretion following experimental *Eimeria acervulina* infection. Chickens were fed from hatch with non-supplemented or PTSO/PTS-supplemented diets and either uninfected or orally infected with $1{\cdot}0 \times 10^4$ oocysts of *E. acervulina* at 10 d post-hatch. (A) Body weights (twenty birds/group) were measured in non-infected and infected chickens on the non-supplemented diet (control), and in infected chickens on the PTSO/PTS-supplemented diet at 0 and 10 d post-infection. Values are means, with standard deviations represented by vertical bars. [a,b] Mean values with unlike letters were significantly different according to Duncan's multiple-range test ($P<0{\cdot}05$). (B) Faecal samples (twenty birds/group) were collected from chickens on the non-supplemented (control) and PTSO/PTS-supplemented diets between 6 and 9 d post-infection and total oocyst numbers were determined using a McMaster chamber. Values are means, with standard deviations represented by vertical bars. ** Mean value was significantly different from that of the control group ($P<0{\cdot}01$; Students *t* test).

in the uninfected control at 10 d post-infection, and dietary PTSO/PTS significantly ($P<0{\cdot}05$) increased serum profilin antibody levels (Fig. 3) compared with non-supplemented controls.

Effect of dietary propyl thiosulphinate oxide/propyl thiosulphinate on differential gene expression

Microarray hybridisation analysis using Agilent Technologies' Chicken Gene Expression Microarray which contains 43 803 elements identified 1227 mRNA whose levels were altered >2·0-fold in intestinal IEL of PTSO/PTS-treated chickens compared with non-supplemented controls. Of these, 552 were

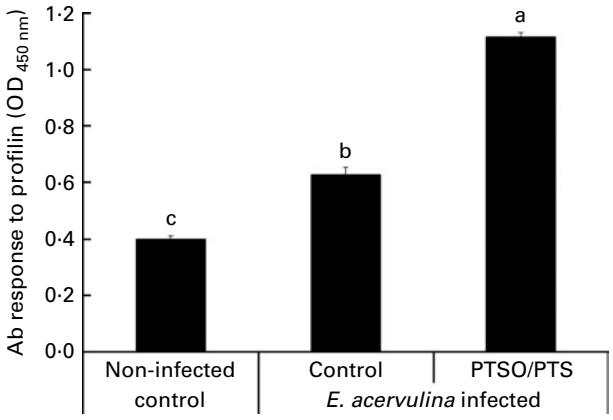

Fig. 3. Effect of dietary propyl thiosulphinate oxide/propyl thiosulphinate (PTSO/PTS) on profilin antibody (Ab) levels. Chickens were fed from hatch with non-supplemented (control) or PTSO/PTS-supplemented diets and orally infected with $1{\cdot}0 \times 10^4$ oocysts of *Eimeria acervulina* at 10 d post-hatch. Peripheral blood (four birds/group) was collected at 10 d post-infection and sera were analysed for anti-profilin Ab levels by ELISA. Values are means, with standard deviations represented by vertical bars (*n* 4). [a,b,c] Mean values with unlike letters are significantly different according to Duncan's multiple-range test ($P<0{\cdot}05$). OD, optical density.

increased and 675 were decreased. This data set was mapped to the corresponding genes of the human, mouse and rat genome using Ingenuity Knowledge Base software (Ingenuity Systems), and 288 chicken genes were identified and annotated. Tables 2 and 3 list the genes corresponding to the twenty most up- or down-regulated transcripts, respectively.

Quantitative RT-PCR validation

The expression patterns observed by microarray analysis were validated by quantitative RT-PCR with four selected transcripts whose levels were significantly modulated between PTSO/PTS-fed and non-treated chickens. These genes were adiponectin receptor 2, aspartate β-hydroxylase, IL-8 and TNF (ligand) superfamily member 13b (*TNFSF13B*). The levels of these transcripts that were up- or down-regulated by microarray hybridisation were also correspondingly altered when analysed by quantitative RT-PCR (Fig. 4). As previously discussed, the differences in the magnitude between the changes observed, particularly with adiponectin receptor 2 and aspartate β-hydroxylase, might be due to differences in the normalisation methods and/or the different fluorescent dyes used in the two techniques[31].

Biological function and pathway analyses

Biological function analysis using the IPA (Ingenuity Systems) database was performed for the 288 genes corresponding to the mRNA that were differently altered following dietary PTSO/PTS treatment compared with untreated controls. The *P* value associated with a particular function in this analysis is a statistical measure of the likelihood that genes from the data set under investigation participate in that function. In this manner, these genes were classified under the categories 'Disease and Disorders' and 'Physiological System Development

Table 2. Genes corresponding to up-regulated intestinal intraepithelial lymphocyte transcripts in chickens given a propyl thiosulphinate oxide/propyl thiosulphinate-supplemented diet compared with a non-supplemented diet

Symbol	Entrez gene name	Agilent no.	Fold change	Location	Function
PDPR	Pyruvate dehydrogenase phosphatase regulatory subunit	A_87_P008075	69·47	Unknown	Enzyme
ITGB1BP2	Integrin beta 1 binding protein (melusin) 2	A_87_P024694	46·814	Unknown	Other
SH3BP4	SH3-domain binding protein 4	A_87_P007874	38·585	Unknown	Other
CRH	Corticotropin-releasing hormone	A_87_P037220	32·849	Extracellular space	Other
SDK2	Sidekick homologue 2 (chicken)	A_87_P037566	23·605	Unknown	Other
MDK	Midkine (neurite growth-promoting factor 2)	A_87_P009319	23·142	Extracellular space	Growth factor
C6ORF167	Chromosome 6 open reading frame 167	A_87_P037304	21·443	Unknown	Other
TMEM213	Transmembrane protein 213	A_87_P021765	20·831	Unknown	Other
C9ORF24	Chromosome 9 open reading frame 24	A_87_P020203	17·712	Unknown	Other
HBE1	Hb, epsilon 1	A_87_P032051	16·162	Cytoplasm	Transporter
TRRAP	Transformation/transcription domain-associated protein	A_87_P000649	14·944	Nucleus	Transcription regulator
CBX6-NPTXR	Cbx6-Nptxr readthrough transcripts	A_87_P040723	12·795	Unknown	Other
MACF1	Microtubule-actin crosslinking factor 1	A_87_P011329	12·455	Cytoplasm	Other
AGXT2L1	Ala-glyoxylate aminotransferase 2-like 1	A_87_P016602	12·318	Unknown	Enzyme
CDON	Cdon homologue (mouse)	A_87_P015827	11·982	Plasma membrane	Other
VAX1	Ventral anterior homeobox 1	A_87_P038172	11·949	Nucleus	Transcription regulator
VTN	Vitronectin	A_87_P008745	11·378	Extracellular space	Other
ARC	Activity-regulated cytoskeleton-associated protein	A_87_P037475	11·005	Cytoplasm	Other
POMC	Pro-opiomelanocortin	A_87_P009245	10·626	Extracellular space	Other

Table 3. Genes corresponding to down-regulated intestinal intraepithelial lymphocyte transcripts in chickens given a propyl thiosulphinate oxide/propyl thiosulphinate-supplemented diet compared with a non-supplemented diet

Symbol	Entrez gene name	Agilent no.	Fold change	Location	Function
UCP3	Uncoupling protein 3 (mitochondrial, proton carrier)	A_87_P037720	0·063	Cytoplasm	Transporter
F2RL2	Coagulation factor II (thrombin) receptor-like 2	A_87_P021894	0·088	Plasma membrane	G-protein coupled receptor
FCN2	Ficolin (collagen/fibrinogen domain containing lectin) 2 (hucolin)	A_87_P012535	0·102	Extracellular space	Other
LAMB2	Laminin, beta 2 (laminin S)	A_87_P037980	0·127	Extracellular space	Enzyme
C1ORF63	Chromosome 1 open reading frame 63	A_87_P023430	0·143	Unknown	Other
NUF2	NUF2, NDC80 kinetochore complex component, homologue (*S. cerevisiae*)	A_87_P036083	0·154	Nucleus	Other
FREM1	FRAS1 related extracellular matrix 1	A_87_P029906	0·154	Extracellular space	Other
C5ORF34	Chromosome 5 open reading frame 34	A_87_P016635	0·165	Unknown	Other
TLR5	Toll-like receptor 5	A_87_P018174	0·171	Plasma membrane	Transmembrane receptor
HMGCS2	3-Hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	A_87_P031965	0·171	Cytoplasm	Enzyme
MTMR7	Myotubularin related protein 7	A_87_P011372	0·176	Cytoplasm	Phosphatase
PDCD2L	Programmed cell death 2-like	A_87_P036130	0·190	Unknown	Other
C21ORF7	Chromosome 21 open reading frame 7	A_87_P023657	0·191	Unknown	Other
SCN2A	Sodium channel, voltage-gated, type II, α subunit	A_87_P015799	0·194	Plasma membrane	Ion channel
C8ORF4	Chromosome 8 open reading frame 4	A_87_P024345	0·196	Unknown	Other
C13ORF27	Chromosome 13 open reading frame 27	A_87_P021548	0·203	Unknown	Other
WDR47	WD repeat domain 47	A_87_P034462	0·203	Unknown	Other
ERI1	Exoribonuclease 1	A_87_P036756	0·203	Unknown	Enzyme
PNLIP	Pancreatic lipase	A_87_P023861	0·211	Extracellular space	Enzyme
CREBL2	cAMP responsive element binding protein-like 2	A_87_P021605	0·212	Nucleus	Transcription regulator

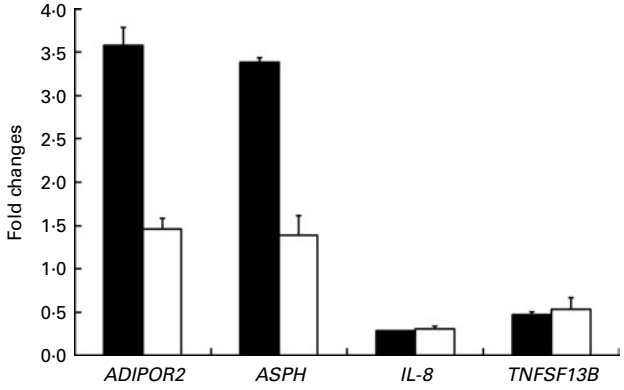

Fig. 4. Comparison between microarray (■) analysis and quantitative RT-PCR (□) for the levels of mRNA corresponding to selected genes. Each bar represents fold changes of mRNA levels in propyl thiosulphinate oxide/propyl thiosulphinate-fed chickens compared with non-supplemented controls. *ADIPOR2*, adiponectin receptor 2; *ASPH*, aspartate β-hydroxylase; *TNFSF13B*, TNF (ligand) superfamily, member 13b.

and Function'. In the category 'Disease and Disorders', the most significant function that was identified was 'Inflammatory Response' (Table 4). In the category 'Physiological System Development and Function', the most significant function that was identified was 'Cardiovascular System Development and Function' (Table 5). Canonical Pathway analysis classified the genes whose expressions were significantly modified by PTSO/PTS dietary supplementation to eight selected biological functions (Table 6). Of these, the pathway of 'Communication Between Innate and Adaptive Immune Cells' was identified as the most significant pathway and included five genes. Overall, seventeen groups of pathway networks, classified according to the number of focus genes, are listed in Table 7. Fig. 5 illustrates the most significant network converging on the NF-κB transcription factor with twenty-four focus genes identified.

Discussion

The present study was conducted to investigate the immunologic and genomic alterations following dietary supplementation with PTSO/PTS, the secondary metabolites of garlic, during *E. acervulina* infection of chickens.

PTSO/PTS enhanced the *in vitro* and *in vivo* parameters of immunity. Treatment of chicken spleen cells with PTSO/PTS increased their proliferation and treatment of *E. acervulina* sporozoites with PTSO/PTS decreased cell viability. Chickens given a PTSO/PTS-supplemented diet and infected with *E. acervulina* had greater body weight gain, reduced faecal oocyst excretion and increased profilin antibody responses compared with chickens fed a non-supplemented diet. Some of these responses may have been due to a direct cytotoxic effect of PTSO/PTS on parasite sporozoites. Microarray hybridisation identified 1227 transcripts whose levels were significantly altered in the intestinal IEL of PTSO/PTS-fed birds compared with non-supplemented controls. Biological pathway analysis identified the altered transcripts as belonging to the categories 'Disease and Disorder' and 'Physiological System Development and Function'.

Garlic has been traditionally used as a folk remedy to treat a variety of human diseases, the health-promoting effects of which are attributed to the presence of multiple sulphur-containing compounds, such as diallyl sulphide, diallyl disulphide, dipropyl sulphide and allicin. These compounds, and others, have demonstrated pharmacological activity *in vitro* and *in vivo*[32–35]. Allicin is generally considered to be one of the primary active compounds that gives garlic its characteristic odour and many of its healing benefits. Thiacremonone, a novel sulphur compound isolated from garlic, possesses anti-inflammatory properties through the inhibition of NF-κB activation in rats and mice[36]. NF-κB is a major transcription factor that plays a key role in regulating the immune response to infection following the binding of pathogenic micro-organisms to a variety of cell surface receptors, such as Toll-like receptors (TLR)[37]. Our bioinformatic analysis identified NF-κB as a central molecule in the most significant network that was activated in PTSO/PTS-treated chickens. This network also included TLR3 and TLR5 as down-regulated components. Further evidence for the modulation of TLR and NF-κB in response to garlic comes from the study of Youn *et al.*[38] showing that the ethyl acetate fraction of the plant bulb inhibited lipopolysaccharide-induced dimerisation of TLR4 and blocked NF-κB activation.

Interestingly, however, our functional studies indicated that dietary PTSO/PTS increased the resistance of chickens to experimental *E. acervulina* infection, as evidenced by greater weight gain and reduced parasite excretion, and augmented parameters of adaptive immunity, including a higher profilin antibody response and greater spleen cell proliferation, compared with chickens fed a non-supplemented diet. The ability of PTSO/PTS to directly kill sporozoites and stimulate spleen cell proliferation *in vitro* suggests that a similar mechanism may operate *in vivo*, thus contributing to increased body weight and decreased parasite fecundity.

The capability of the PTSO/PTS preparation to stimulate spleen cell mitogenesis (an indicator of adaptive cellular immunity), while simultaneously down-regulating TLR and NF-κB (indicators of innate immunity), suggests that the dietary supplement may have more than one active component. In this regard, Clement *et al.*[39] identified several immunomodulatory proteins from garlic with mitogenic activity towards human peripheral blood lymphocytes and murine splenocytes. Further studies to characterise the active components of garlic and their effects on innate and adaptive immunity will help to resolve these questions.

In the biological functional analysis, eighteen genes were related with the most reliable 'Inflammatory Response' category (Table 4). Among those genes, *TLR* are a major component of the pattern recognition receptor repertoire that detect invading micro-organisms and direct the vertebrate immune system to eliminate infection. *TLR5* plays a role in restricting the entry of flagellated *Salmonella* into systemic sites[40] and *TLR3* which recognises double-stranded RNA analogue, induces interferon-β through the recognition of viral double-stranded RNA in chicken[41]. In the present study, *TLR3* and *TLR5* expression was down-regulated in the bioinfomatical analysis and this may explain the reduced inflammatory response

Table 4. Significant functions in the category 'Disease and Disorders' in chickens given a propyl thiosulphinate oxide/propyl thiosulphinate-supplemented diet compared with a non-supplemented diet

Category*	*P* †	Genes
Inflammatory response	$2{\cdot}30\times10^{-3}$–$4{\cdot}24\times10^{-2}$	*UNC13D*, *IL8*, *VCAM1*, *VTN*, *CRH*, *POMC*, *JAK2*, *RNASEL*, *SELP*, *TLR5*, *KL*, *PLCG2*, *MDK*, *HPGDS*, *TLR3*, *ADIPOR2*, *TFPI*, *TNFSF13B*
Dermatological diseases and conditions	$4{\cdot}11\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*IL8*, *SELP*, *KL*, *HBEGF*, *TNFRSF8*, *DSP*
Endocrine system disorders	$4{\cdot}11\times10^{-3}$–$4{\cdot}11\times10^{-3}$	*UBR1*, *CCKBR*
Inflammatory disease	$4{\cdot}11\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*IL8*, *VCAM1*, *SELP*, *TLR5*, *ICA1*, *PDE5A*, *HBEGF*, *TLR3*, *TNFRSF8*, *TNPO3*, *TNFSF13B*
Organismal injury and abnormalities	$4{\cdot}11\times10^{-3}$–$4{\cdot}75\times10^{-2}$	*SOCS3*, *IL8*, *F2RL2*, *SELP*, *VTN*, *UBR1*, *CRH*, *PDE5A*, *HBEGF*, *MDK*, *HPGDS*, *CCKBR*
Renal and urological disease	$4{\cdot}11\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*VCAM1*, *SELP*, *PDE5A*, *HBEGF*, *TLR3*, *MDK*, *TNFSF13B*, *ZNF365*
Genetic disorder	$8{\cdot}01\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*SLC4A4*, *HCRT*, *SGCE*, *DNAJC19*, *CACNB4*, *CRH*, *CACNA1H*, *POMC*, *JAK2*, *HMGCS2*, *CEP290*, *MAOB*, *TLR5*, *LGI1*, *ATCAY*, *DSP*, *ATN1*, *ZNF365*
Infection mechanism	$8{\cdot}01\times10^{-3}$–$4{\cdot}24\times10^{-2}$	*IL8*, *TLR5*, *PLCG2*, *CD93*, *TLR3*
Infectious disease	$8{\cdot}01\times10^{-3}$–$4{\cdot}24\times10^{-2}$	*IL8*, *TLR5*, *PLCG2*, *CD93*, *TLR3*
Metabolic disease	$8{\cdot}01\times10^{-3}$–$4{\cdot}91\times10^{-2}$	*SOCS3*, *PLIN1*, *ICA1*, *DNAJC19*, *CRH*, *HBEGF*, *POMC*, *HMGCS2*, *LAMB2*, *SCP2*, *TLR3*, *ADIPOR2*, *CCKBR*, *ZNF365*
Neurological disease	$8{\cdot}01\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*CA3*, *SCN2A*, *MAOB*, *HCRT*, *SGCE*, *ABCC5*, *LGI1*, *CACNB4*, *CACNA1H*, *ATCAY*, *HPGDS*, *ATN1*
Nutritional disease	$1{\cdot}14\times10^{-2}$–$4{\cdot}75\times10^{-2}$	*UCP3*, *HCRT*, *POMC*, *CCKBR*
Psychological disorders	$1{\cdot}14\times10^{-2}$–$4{\cdot}75\times10^{-2}$	*UCP3*, *HCRT*, *POMC*, *CCKBR*
Gastrointestinal disease	$1{\cdot}14\times10^{-2}$–$4{\cdot}00\times10^{-2}$	*SOCS3*, *CTRC*, *PIAS2*, *DNMT3A*, *CUBN*, *LRRC1*, *HLA-DRB1*, *SYNE1*, *TUFT1*, *KLHL20*, *NSF*, *CA3*, *KIF9*, *KCTD20*, *LNPEP*, *UBXN4*, *EFNA5*, *HPGD*, *TNFSF15*, *RERE*, *ASPH*, *TLR3*, *FABP3*, *CCKBR*, *IL8*, *JAZF1*, *ICA1*, *SEC16A*, *MEF2A*, *MYPN*, *TPD52*, *ITPR1*, *NFASC*, *REV3L*, *FREM1*, *SH3BP5*, *HOPX*, *SELP*, *EDEM2*, *TLR5*, *SCP2*, *REEP3*, *PDE5A*, *TRANK1*, *ZNF365*
Hepatic system disease	$1{\cdot}14\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*JAZF1*, *CUBN*, *EFNA5*, *TUFT1*, *MYPN*, *ITPR1*, *REV3L*, *TLR3*
Connective tissue disorders	$1{\cdot}15\times10^{-2}$–$2{\cdot}60\times10^{-2}$	*VCAM1*, *PLIN1*, *SELP*, *TLR5*, *ICA1*, *TNPO3*, *TNFSF13B*, *CCKBR*
Immunological disease	$1{\cdot}15\times10^{-2}$–$1{\cdot}15\times10^{-2}$	*VCAM1*, *SELP*, *TLR5*, *ICA1*, *TNPO3*, *TNFSF13B*
Skeletal and muscular disorders	$1{\cdot}15\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*VCAM1*, *SELP*, *TLR5*, *ICA1*, *MEF2A*, *TNPO3*, *TNFSF13B*
CVD	$1{\cdot}30\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*F2RL2*, *VCAM1*, *VTN*, *TFPI*, *DSP*
Haematological disease	$1{\cdot}30\times10^{-2}$–$4{\cdot}75\times10^{-2}$	*SOCS3*, *IL8*, *F2RL2*, *SELP*, *SCP2*, *POMC*, *JAK2*, *TFPI*
Antimicrobial response	$2{\cdot}60\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*NCOR1*, *TLR3*, *RNASEL*
Developmental disorder	$2{\cdot}60\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*CEP290*, *PLIN1*, *REV3L*, *ASPH*, *TLR3*, *DSP*, *CCKBR*
Cancer	$3{\cdot}38\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*DDX5*, *LMO4*, *KL*, *HBEGF*, *JAK2*, *ASPH*
Hypersensitivity response	$3{\cdot}38\times10^{-2}$–$4{\cdot}24\times10^{-2}$	*IL8*, *VCAM1*, *SELP*, *TLR3*
Respiratory disease	$3{\cdot}75\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*TLR5*, *PDE5A*

* Data sets were analysed by BioFunction analysis using Ingenuity Pathway Analysis software (Ingenuity Systems).
† *P*-value ranges of the biological functions included in the category. *P* values were calculated using the right-tailed Fisher's exact test.

Table 5. Significant functions in the category 'Physiological System Development and Function' in chickens given a propyl thiosulphinate oxide/propyl thiosulphinate-supplemented diet compared with a non-supplemented diet

Category*	*P* †	Genes
Cardiovascular system development and function	$1{\cdot}21\times10^{-4}$–$4{\cdot}86\times10^{-2}$	*IL8*, *VCAM1*, *GEM*, *VTN*, *MEF2A*, *ANGPTL2*, *CRH*, *POMC*, *DICER1*, *JAK2*, *KLHL20*, *MLL*, *SELP*, *TNFSF15*, *MDK*, *ASPH*, *TFPI*
Nervous system development and function	$9{\cdot}61\times10^{-4}$–$4{\cdot}22\times10^{-2}$	*F2RL2*, *VAX1*, *VCAM1*, *HCRT*, *GEM*, *LMO4*, *CACNA1H*, *CRH*, *DLX1*, *POMC*, *JAK2*, *BCL11B*, *LAMB2*, *SELP*, *EFNA5*, *CDON*, *HPGDS*, *TLR3*
Embryonic development	$1{\cdot}40\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*SOCS3*, *VAX1*, *VCAM1*, *CTCF*, *GDF3*, *KL*, *MYST3*, *SEMA6D*, *HBEGF*, *GREM1*, *TRRAP*, *MLL*
Behaviour	$1{\cdot}64\times10^{-3}$–$4{\cdot}24\times10^{-2}$	*HCRT*, *SLC16A2*, *CRH*, *POMC*
Haematological system development and function	$1{\cdot}99\times10^{-3}$–$4{\cdot}75\times10^{-2}$	*IL8*, *SOCS3*, *VCAM1*, *VTN*, *CDK6*, *CRH*, *HLA-DRB1*, *POMC*, *JAK2*, *MLL*, *KCNK5*, *TLR5*, *SELP*, *MYST3*, *TNFSF15*, *TNFRSF8*, *TLR3*, *WASF2*, *MDK*, *TNFSF13B*
Haematopoiesis	$1{\cdot}99\times10^{-3}$–$4{\cdot}44\times10^{-2}$	*SOCS3*, *IL8*, *VCAM1*, *SELP*, *MYST3*, *CDK6*, *CRH*, *POMC*, *JAK2*, *MLL*, *TNFSF13B*
Organismal development	$1{\cdot}99\times10^{-3}$–$4{\cdot}94\times10^{-2}$	*SOCS3*, *IL8*, *VCAM1*, *PLIN1*, *GDF3*, *CDK6*, *CRH*, *UBR1*, *POMC*, *JAK2*, *MLL*, *KLHL20*, *LAMB2*, *KCNK5*, *UCP3*, *MAOB*, *SELP*, *MYST3*, *TLR3*
Immune cell trafficking	$2{\cdot}30\times10^{-3}$–$4{\cdot}44\times10^{-2}$	*IL8*, *VCAM1*, *TLR5*, *SELP*, *VTN*, *CRH*, *POMC*, *TLR3*, *MDK*, *JAK2*, *TNFSF13B*
Tissue development	$2{\cdot}30\times10^{-3}$–$4{\cdot}75\times10^{-2}$	*IL8*, *SOCS3*, *VAX1*, *VCAM1*, *GDF3*, *VTN*, *UBR1*, *GREM1*, *POMC*, *NFASC*, *JAK2*, *SELP*, *KL*, *TLR3*, *TFPI*, *PRKD1*, *TNFSF13B*
Connective tissue development and function	$4{\cdot}11\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*VCAM1*, *UCP3*, *PLIN1*, *KL*, *UBR1*, *CRH*, *POMC*
Tissue morphology	$4{\cdot}11\times10^{-3}$–$4{\cdot}75\times10^{-2}$	*SOCS3*, *VCAM1*, *PLIN1*, *LMO4*, *VTN*, *CRH*, *UBR1*, *POMC*, *UCP3*, *SELP*, *KL*, *TNFSF13B*, *CCKBR*
Cell-mediated immune response	$6{\cdot}90\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*SOCS3*, *IL8*, *VCAM1*, *SELP*, *TNFSF13B*
Endocrine system development and function	$8{\cdot}01\times10^{-3}$–$4{\cdot}24\times10^{-2}$	*DBI*, *COMT*, *CRH*, *POMC*, *CCKBR*
Organ morphology	$8{\cdot}01\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*VCAM1*, *SELP*, *MEF2A* (includes *EG:4205*), *CRH*, *HBEGF*, *POMC*, *DICER1*, *TNFSF13B*, *CCKBR*
Organismal functions	$8{\cdot}01\times10^{-3}$–$3{\cdot}75\times10^{-2}$	*IL8*, *MAOB*, *HCRT*, *COMT*, *CRH*
Skeletal and muscular system development and function	$1{\cdot}30\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*LAMB2*, *SOCS3*, *HOPX*, *PLIN1*, *LMO4*, *KL*, *VTN*, *CDON*, *MDK*, *MYOG*, *TFPI*, *BARX2*
Lymphoid tissue structure and development	$1{\cdot}32\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*IL8*, *SELP*, *VTN*, *MDK*, *TFPI*, *TNFSF13B*
Reproductive system development and function	$1{\cdot}32\times10^{-2}$–$4{\cdot}24\times10^{-2}$	*SOCS3*, *NSF*, *VCAM1*, *GNPDA1*, *LNPEP*, *COMT*, *CRH*, *HPGD*, *HBEGF*
Digestive system development and function	$1{\cdot}91\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*HCRT*, *CRH*, *CCKBR*
Organ development	$3{\cdot}38\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*SOCS3*, *IL8*, *VAX1*, *SELP*, *CRH*, *POMC*
Auditory and vestibular system development and function	$3{\cdot}75\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*IL8*
Hair and skin development and function	$3{\cdot}75\times10^{-2}$–$4{\cdot}24\times10^{-2}$	*IL8*, *CTCF*, *ABCC5*, *POMC*, *JAK2*, *DSP*
Humoral immune response	$3{\cdot}75\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*TNFSF13B*
Organismal survival	$3{\cdot}75\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*TLR3*
Renal and urological system development and function	$3{\cdot}75\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*KCNK5*, *GEM*, *CTCF*, *CDK6*, *GREM1*
Respiratory system development and function	$3{\cdot}75\times10^{-2}$–$3{\cdot}75\times10^{-2}$	*GREM1*, *DICER1*
Tumour morphology	$3{\cdot}75\times10^{-2}$–$4{\cdot}75\times10^{-2}$	*SOCS3*, *VCAM1*, *VTN*, *NFASC*, *TFPI*

* Data sets were analysed by BioFunction analysis using Ingenuity Pathway Analysis software (Ingenuity Systems).
† *P*-value ranges of the biological functions included in the category. *P* values were calculated using the right-tailed Fisher's exact test.

Table 6. Significant canonical pathways in chickens given a propyl thiosulphinate oxide/propyl thiosulphinate-supplemented diet compared with a non-supplemented diet

Canonical pathway*	*P*†	Ratio‡	Focus genes
Communication between innate and adaptive immune cells	$1{\cdot}38\times10^{-3}$	$4{\cdot}67\times10^{-2}$	*IL-8*, *TLR5*, *HLA-DRB1*, *TLR3*, *TNFSF13B*
Corticotropin-releasing hormone signalling	$2{\cdot}04\times10^{-3}$	$5{\cdot}88\times10^{-2}$	*PLCG2*, *GNAI1*, *CRH*, *MEF2A* (includes *EG:4205*), *POMC*, *ITPR1*, *ADCY7*, *PRKD1*
TREM1 signalling	$4{\cdot}07\times10^{-3}$	$7{\cdot}25\times10^{-2}$	*IL-8*, *TLR5*, *PLCG2*, *JAK2*, *TLR3*
α-Adrenergic signalling	$9{\cdot}55\times10^{-3}$	$5{\cdot}66\times10^{-2}$	*PHKB*, *PLCG2*, *GNAI1*, *ITPR1*, *ADCY7*, *PRKD1*
Altered T-cell and B-cell signalling in rheumatoid arthritis	$1{\cdot}95\times10^{-2}$	$4{\cdot}40\times10^{-2}$	*TLR5*, *HLA-DRB1*, *TLR3*, *TNFSF13B*
Glutathione metabolism	$2{\cdot}45\times10^{-2}$	$4{\cdot}08\times10^{-2}$	*GSTT1*, *LNPEP*, *ACSS2*, *HPGDS*
Cyanoamino acid metabolism	$2{\cdot}63\times10^{-2}$	$3{\cdot}12\times10^{-2}$	*KL*, *ACSS2*
Leptin signalling in obesity	$3{\cdot}80\times10^{-2}$	$5{\cdot}95\times10^{-2}$	*SOCS3*, *PLCG2*, *POMC*, *JAK2*, *ADCY7*

TREM1, triggering receptor expressed on myeloid cells 1.
* Data sets were analysed by Bio Function analysis using Ingenuity Pathway Analysis software (Ingenuity Systems).
† *P* values were calculated using the right-tailed Fisher's exact test.
‡ The ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway.

Table 7. Significant networks in chickens given a propyl thiosulphinate oxide/propyl thiosulphinate-supplemented diet compared with a non-supplemented diet

ID	Associated network functions	No. of focus genes	Score
1	Cellular compromise, cell-to-cell signalling and interaction, cellular growth and proliferation	24	38
2	Cardiovascular system development and function, lipid metabolism, small-molecule biochemistry	21	32
3	Connective tissue development and function, embryonic development, skeletal and muscular system development and function	19	29
4	Cell morphology, cellular development, cellular movement	21	28
5	Cardiovascular system development and function, cell morphology, cellular development	19	28
6	Carbohydrate metabolism, small-molecule biochemistry, lipid metabolism	17	24
7	Endocrine system development and function, nervous system development and function, tissue morphology	16	21
8	Lipid metabolism, small-molecule biochemistry, amino acid metabolism	15	20
9	Free-radical scavenging, genetic disorder, metabolic disease	14	19
10	Cell signalling, nucleic acid metabolism, small-molecule biochemistry	14	19
11	Gene expression, cellular compromise, connective tissue disorders	13	17
12	Cell cycle, cellular development, gene expression	13	16
13	Organismal functions, haematological system development and function, tissue morphology	12	15
14	Cardiovascular system development and function, molecular transport, small-molecule biochemistry	12	15
15	DNA replication, recombination, and repair, energy production, nucleic acid metabolism	12	15
16	Molecular transport, small-molecule biochemistry, cell-to-cell signalling and interaction	12	15
17	Cell cycle, DNA replication, recombination, and repair, cellular growth and proliferation	12	14

seen at the later phase of coccidiosis infection. Although the underlying mechanisms are not yet known, it was found that garlic increases NK cell activity and T cell proliferation in mice[42]. Aged garlic extract treatment enhanced NK cell activity[43,44] and phagocytosis[5]. The balance of T helper 1/T helper 2-type cytokines, such as the secretion of interferon-γ and IL-2 *v.* IL-4 and IL-10, was regulated by the consumption of garlic products in a dose-dependent manner[45–47]. In chickens, IEL contains two major phenotypically and functionally distinct populations, NK and T cells, and these cells play important roles in avian coccidiosis[17]. Therefore, it is possible that dietary garlic enhanced disease resistance against coccidiosis by activating these effector cells and enhancing the secretion of immunomodulating cytokines in the gut. The TNF family is an important regulator of inflammation, immune responses and tissue homeostasis[48]. TNFSF13B, also known as B-cell activating factor (B-cell activating factor of the TNF family), plays a major role in B-cell survival, proliferation and differentiation. TNFSF13B induced the selective expansion of B cells in the spleen and caecal tonsils when administered to young chicks[49]. The level of TNFSF13B was shown to increase during various diseases that may reflect the severity of disease in malaria, virus infection and multiple sclerosis[50–52]. However, the overexpression of TNFSF13B is also involved in some autoimmune conditions[53,54]. In the present study, dietary feeding of normal chickens with PTSO/PTS enhanced serum antibody response to *E. acervulina* infection in *Eimeria*-infected chickens when antibodies were measured at 10 d after infection. However, for gene expression analysis, we used uninfected gut tissues from chickens treated with PTSO/PTS, and we saw the decreased TNFSF13B transcript compared to the chickens that were not fed with PTSO/PTS. Therefore, we speculate that the expression of TNFSF13B may have increased upon coccidiosis infection and this may have caused high antibody response. Chicken IL-8 (IL-8/CXCLi2) was the first chicken CXC chemokine identified and significantly triggered by the exposure of the gut to feed and bacteria in newly hatched chicks[55]. IL-8 has both chemotactic and angiogenic functions in the chicken[56]; so this gene was also identified in the 'Cardiovascular System Development

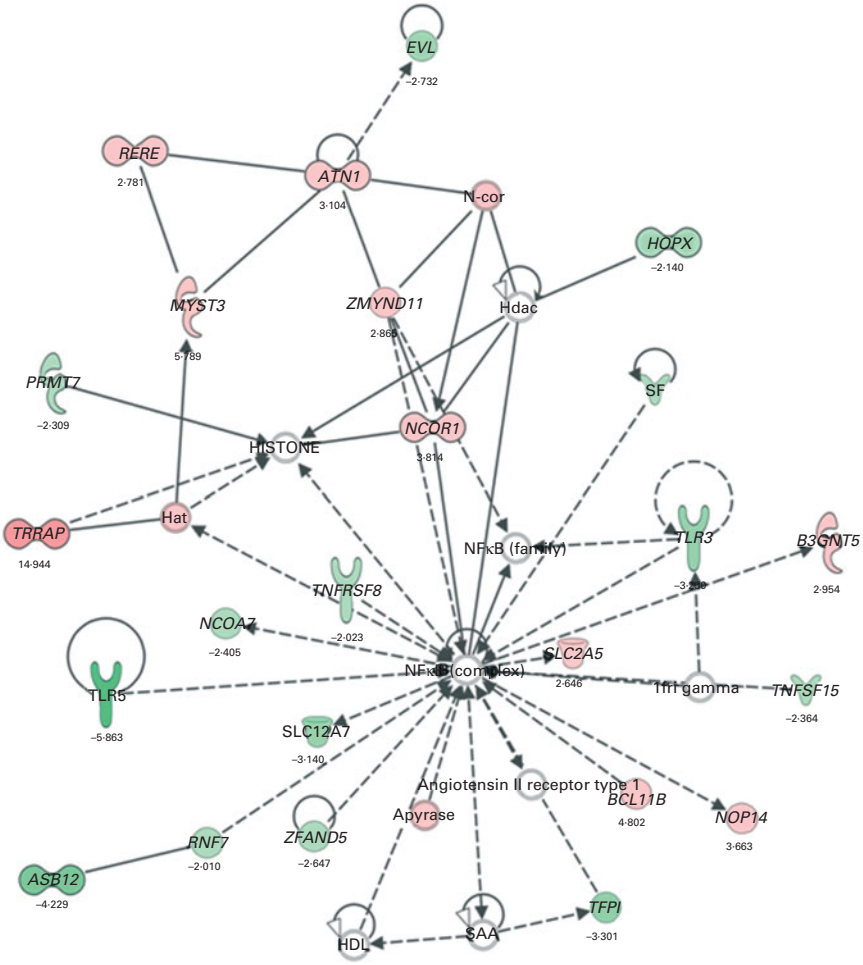

Fig. 5. The most significant network with the indicated genes corresponding to mRNA exhibiting >2·0-fold up- and down-regulated levels following dietary supplementation of propyl thiosulphinate oxide/propyl thiosulphinate compared with non-supplemented controls. Genes corresponding to increased and decreased mRNA are indicated by red and green colours, respectively, with the colour intensity indicating the relative expression levels. Overlaid numbers are the fold changes associated with each gene. ○, Complex; ⩛, cytokine/growth factor; ⬮, enzyme; ◍, group/complex/other; ⋈, transcription regulator; ⩛, transmembrane receptor; ▽, transporter; ○, unknown; ▬, direct relationship; --, indirect relationship.

and Function' category (Table 5). At low concentrations, IL-8 is chemotactic for monocyte/macrophages and lymphocytes. However, at high concentrations, IL-8 stimulates sprouting and growth of blood vessels[57].

In addition to effects on inflammation and immunity, dietary garlic dramatically affects the cardiovascular system[58]. For example, garlic consumption by humans may diminish the progression of CVD by decreasing the levels of LDL and total cholesterol, while concomitantly raising HDL levels. Consumption of garlic is also associated with the inhibition of platelet aggregation and reduced systolic and diastolic blood pressure. Recently, garlic was found to decrease two other markers of CVD, homocysteine and C-reactive protein. Interestingly, our present results identified 'Cardiovascular System Development and Function' as the most significant function in the category 'Physiological System Development and Function' of PTSO/PTS-fed chickens. This finding may extend the effects of garlic metabolites to poultry. However, the underlying pathways associated with the cardiovascular system in poultry still needs to be studied.

In conclusion, *in vivo* feeding of young broiler chickens with PTSO/PTS compound, a garlic-derived extract, improved resistance to experimental *E. acervulina* infection and induced significant alterations of the transcriptome in chicken intestinal lymphocytes involving immune- and cardiovascular-related gene pathways and networks. These results suggest that dietary immunomodulation by garlic-derived compounds may represent a possible alternative to current drug-based strategies for commercial poultry production.

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