Original article

Immunological and gene expression responses to a *Salmonella* infection in the chicken intestine

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Abstract – Besides infection in humans, *Salmonella enteritidis* can also cause serious illness in young chickens. However, the genetic and immunological parameters important for the disease in chickens are not well characterized. In this study, processes in the chicken intestine in response to a *Salmonella* infection were investigated in two different chicken lines. One-day-old chickens were orally infected with *Salmonella*. T-cell subpopulations, phagocytic properties of intestinal mononuclear cells and RNA expression levels of the jejunum were investigated. The two chicken lines differed in the amount of cfu in the liver and growth retardation after the infection. Differences in phagocytic activity of intestinal mononuclear cells were found between control and *Salmonella* infected chickens. The number of CD4$^+$ T-cells of the intestine decreased after the *Salmonella* infection in one chicken line, while the number of CD8$^+$ T-cells increased in both chicken lines, but the time post infection of this increase differed between the lines. In one chicken line the expression levels of the genes carboxypeptidase M and similar to ORF2 decreased after the *Salmonella* infection, which might be related to a decrease in the amount of macrophages. With the microarray, ten genes were found that were regulated in only one of the chicken lines, while we found six genes regulated in response to the infection in both chicken lines. So differences in genetic background of the chickens influence the intestinal host response of the *Salmonella* infection as observed by phagocytic activity, gene expression and changes in the number of T-cell subpopulations and macrophages.

immunology / microarray / *Salmonella* / T-cell

1. INTRODUCTION

Salmonella enterica is one of the most common causes of food poisoning in humans, mostly caused by poultry products infected by *S. enterica* serovars Typhimurium or Enteritidis [16]. In addition to the enteric disease in humans, *Salmonella* serovars Typhimurium and Enteritidis are also capable of causing severe systemic disease in newly hatched chicks and in birds under extreme stress conditions [19]. In young chickens, infection with *Salmonella* leads to diarrhea and intestinal lesions and to an influx of heterophils into the gut accompanied by inflammation and damage to villi [3].

An infection with *Salmonella* usually starts by ingestion, followed by colonization in the intestine. After colonization, *Salmonella* is able to penetrate the

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mucosal epithelium which results in a systemic infection, with colonization of the spleen and liver [9]. The immunological responses in the chicken intestine to a *Salmonella* infection are not fully understood. In general, the innate immune system plays an important role in the early response to *Salmonella*. Upon oral challenge with *Salmonella* the area occupied by the macrophages in the caecal wall was increased in neonatal chickens [24]. Macrophage-derived cytokines and immune mediators can initiate local and systemic inflammatory responses. This local inflammation likely explains the strong influx and continued presence of macrophages and T-cells after a *Salmonella* infection [24]. It was also shown that after infection with *Salmonella* the number of CD8$^+$TCR$\gamma\delta^+$ T-cells in the chicken intestine increased [6]. CD4$^+$ or CD8$^+$ T-cell responses are not required for the early host response [14], but CD4$^+$ T-cell responses, particularly Th1 responses, play an important role in the clearance of *Salmonella* from the gastrointestinal tract [5, 10, 27].

Earlier gene expression studies suggested that innate immunity, inflammation and T-cell responses are important processes in the chicken intestine in response to a *Salmonella* infection [23]. However, these findings were only based on gene expression data of one chicken experiment. To further evaluate immune responses in the one-day-old chicken intestine after a *Salmonella* infection, differences in T-cell populations were investigated as were phagocytic properties of intestinal mononuclear cells and RNA expression.

2. MATERIALS AND METHODS

2.1. Chickens

Two meat type chicken lines, a fast growing line F and a slow growing line S were used in the present study (Nutreco®, Boxmeer, The Netherlands). Line S is a commercial dam line from white plymouth rock origin. Line F is an experimental line selected for egg production, liveability and slow growth to be used in the future for processing of broilers of 80 days and older. As indicated in the results section, these lines differed in growth retardation and amount of colony forming units in the liver after *Salmonella* infection. One-day old chickens of each line (F and S) were randomly divided into 2 groups, 40 chickens each. After hatching, birds were checked to be free of *Salmonella*.

2.2. Experimental infection

Salmonella enterica serovar Enteritidis phage type 4 (nalidixic acid resistant) was grown in buffered peptone water (BPW) overnight while shaking. In each chicken line, one group of 1-day old chickens was orally inoculated with 0.2 mL of the bacterial suspension containing 10^5 cfu *S. enterica* serovar Enteritidis. The control groups were inoculated with 0.2 mL saline. Ten chickens of each group were randomly chosen, weighed and sacrificed at days 1, 5 and 7 post infection (pi). Pieces of the jejunum were snap frozen in liquid nitrogen and stored at −70 °C for immunohistochemistry and RNA isolation. The remaining part of the jejunum was stored in buffered saline at 4 °C until isolation of intestinal mononuclear cells. The liver was removed and weighed and kept at 4 °C until bacteriological examination. At day 9 the chickens were weighed only and at day 12 the chickens were weighed and bacteriological examinations were performed, but no mononuclear cells or RNA was isolated. The study was approved by the institutional Animal Experiment Commission in accordance with Dutch regulations on animal experimentation.

2.3. Bacteriological examination

For detection of *S*. serovar Enteritidis a cloacal swab was taken and after overnight enrichment it was spread on brilliant green agar + 100 ppm nalidixic acid for *Salmonella* determination (37 °C, 18−24 h). One gram of liver of each bird was homogenized in 9 mL BPW, serial diluted in BPW, and plated onto brilliant green agar with nalidixic acid for quantitative *S*. serovar Enteritidis determination (37 °C, 18−24 h) by counting the colony forming units. To identify significant differences between the two chicken lines, a student-*t* test was performed on the log-transformed data.

2.4. Intestinal mononuclear cell isolation

The jejunum was opened longitudinally, washed with phosphate buffered saline (PBS) and cut into pieces of 1 cm. These pieces were incubated at 37 °C for 45 min in Medium I (PBS containing 1 mM EDTA and 5 mM DTT). The suspension contained the intraepithelial cells (fraction 1) and was kept at 4 °C until use. The remaining pieces of intestine were further incubated at 37 °C for 90 min in Medium II (RPMI + 5% fetal calf serum (FCS) + 400 FALGPA units Collagenase per liter (Sigma, St. Louis, MO, USA) + 60000 Kunitz units DNase I per liter (Sigma)) while shaking (fraction 2, lamina propria cells). The two fractions were mixed and after centrifugation for 10 min at $460 \times g$ the pellet was resuspended in 10 mL Medium III (RPMI + 1% FCS + 60000 Kunitz units DNase I per liter). The suspension was purified on a 25% percoll (Sigma) gradient centrifuging for 15 min at 2000 rpm. The pellet was washed twice with PBS and the cells were coloured with 0.1% trypan blue and viable (unstained) cells were counted. The cells were resuspended in PBS at a concentration of 1×10^6 cells per mL.

2.5. Phagocyte activity of intestinal mononuclear cells

The intestinal mononuclear cell isolates were tested for their phagocytic activity by intake of live *Salmonella enteritidis* phage type 4 as described by Kramer et al. [12]. Briefly, the gut mononuclear cell suspension was diluted to 1×10^7 cells/mL in RPMI. One milliliter of *Salmonella enteritidis* (overnight culture 1:100 diluted and grown for approximately 3 h, about 1×10^8 cfu) was added and the mixture was incubated for 45 min at 37 °C. Subsequently, 200 µg gentamycin was added to kill non-internalized bacteria and incubated for 45 min at room temperature. After washing in PBS the cells were lysed by adding 1 mL 0.2% saponine in PBS and incubating 5 min to release the bacteria internalized by the phagocytic intestinal mononuclear lymphocytes. The number of *S. enteritidis* internalized by the cells was counted on BGA-NaI$^+$ plates. A higher value indicated a higher phagocytic activity of the mononuclear cells.

2.6. Flow cytometry

The total leukocyte subpopulation of the intestinal mononuclear cell isolates was estimated by flow cytometry. For the flow cytometric analysis the concentration of the isolated cells was brought to 20×10^6 cells/mL, and 50 µL was transferred into a 96 well plate on ice. The cells were washed with PBS supplemented with 1% FCS. A normal mouse serum (1%) was applied to block non-specific binding sites, followed by adding the monoclonal antibody CD45-PE (Southern Biotech, Birmingham, Alabama, USA). After 15 min incubation at 4 °C, the cells were washed twice with PBS/FCS and re-suspended in 200 µL ice-cold PBS/FCS. A total of 10^4 cells per sample were analyzed by flow cytometry (FACS Calibur^{TM}, Beckton

Dickinson, Leiden, The Netherlands). The data were analyzed using a flow cytometry computer program.

2.7. Immunohistochemistry

Frozen jejunum sections collected at days 1, 5 and 7 pi were stained for CD4$^+$ T-cells, CD8$^+$ T-cells and macrophages. Immunohistological staining by an indirect immunoperoxidase method was performed on frozen tissue sections (10 μm thick). The sections were loaded on glass slides, air-dried, and fixed in acetone for 10 min. After being dried, the slides were immersed in PBS with 0.1% BSA and were subsequently incubated for 1 h with monoclonal antibodies against macrophages (1:500 CVIChNL68.1 [11]), CD4$^+$ T cells (1:200 CT-4 Southern Biotech), or CD8$^+$ T cells (1:200 CT-8 Southern Biotech) followed by peroxidase-conjugated rabbit anti-mouse Ig (1:80 Dakopatts, Denmark). Peroxidase activity was detected by 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M Tris-HCl solution (pH 7.5) containing 0.03% H$_2$O$_2$. The sections were further colored with 1% CoCl$_2$ for 5 min. After washing, the nuclei were counter-stained with hematoxylin. The sections were dehydrated and mounted in distyrene-tricresyl phosphate-xylene (DPX). The images were acquired and analyzed with Image-Pro Plus (version 5.1, media cybernetics).

2.8. RNA isolation

Pieces of the jejunum were crushed under liquid nitrogen. Fifty to hundred mg tissues of the different chicks were used to isolate total RNA using TRIzol reagent (Invitrogen, Breda, The Netherlands), according to instructions of the manufacturer with an additional step. The homogenized tissue samples were resuspended in 1 mL of TRIzol Reagent using a syringe and 21 gauge needle and passing the lysate through 10 times. After homogenization, insoluble material was removed from the homogenate by centrifugation at 12 000×g for 10 min at 4 °C. For the array hybridization, pools of RNA were made in which equal amounts of RNA from ten different chickens of the same line, condition and timepoint were present.

2.9. Hybridizing of the microarray

The microarrays were constructed as described earlier [21]. The microarrays contained 3072 cDNA from a subtracted intestinal library and 1152 cDNA from a concanavalin A stimulated spleen library. All cDNA were spotted in triplicate on each microarray. Before hybridization, the microarray was pre-hybridized in 5% SSC, 0.1% SDS and 1% BSA at 42 °C for 30 min. To label the RNA, the MICROMAX TSA labeling and detection kit (PerkinElmer, Wellesley, MA, USA) was used. The TSA probe labeling and array hybridization were performed as described in the instruction manual with minor modifications. Biotin- and fluorescein-labeled cDNA were generated from 5 μg of total RNA from the chicken jejunum pools per reaction. The cDNA synthesis time was increased to 3 h at 42 °C. The generated cDNA was transferred to a microcon YM-100 centrifugal filter cartidge (Millipore, Billerica, MA, USA) and washed twice with 10 mM Tris-HCl, pH 7.5. The cDNA was resolved in 60 μL hybridization buffer from the kit and heated to 95 °C for 2 min. Hybridization of the array was done during 16−20 h at 65 °C. Post-hybridization washes were performed according to the manufacturer's recommendations. Hybridizations were performed in duplicate with the fluorophores reversed. After signal amplification, the microarrays were dried and scanned for Cy5 and Cy3 fluorescence in

a Packard Bioscience BioChip Technologies apparatus. The image was processed with Genepix pro 5.0 (Genomic Solutions, Ann Arbor, MI, USA) and the spots were located and integrated with the spotting file of the robot used for spotting. Reports were created of total spot information and spot intensity ratio for subsequent data analyses.

2.10. Analysis of the microarray data

Each spot was corrected for local background and the data for each slide were normalized so that the mean of the ratio of all spots was equal to one with the GenePix Pro 5.0 program. A total of 8 microarrays were used in this experiment. The following four comparisons were made using pools of RNA from ten different chickens:1- line F (fast growing) control vs. line S (slow growing) control; 2-line F *Salmonella* vs. line S *Salmonella*; 3-line F control vs. line F *Salmonella*; and 4-line S control vs. line S *Salmonella*. For each comparison, six values were obtained per gene, three for one slide and three for the dye-swap. Genes with two or more missing values were removed from further analysis. Missing values were possibly due to a bad signal to noise ratio. A gene was considered to be differentially expressed when the mean value of the ratio log2 (Cy5/Cy3) was > 1.58 or < -1.58 and the cDNA was identified with a one class significance analysis of microarrays (based on SAM [20]) with a False discovery rate $< 2\%$. Because the ratio was expressed in a log2 scale, a ratio of > 1.58 or < -1.58 corresponded to a more than threefold up- or downregulation respectively, which is the expression difference limit indicated by the manufacturer of the MICROMAX TSA labeling and detection kit.

2.11. Quantitative real time PCR

A quantitative PCR was performed as described previously [22]. Briefly, 200 ng of total RNA from the jejunum was reverse transcribed with random hexamers. Generated cDNA was stored at –20 °C until use. PCR amplification and analysis was done with the described primers and conditions (Tab. I). 28S was used as a control to correct for the input of cDNA. Each reaction mixture consisted of 1 μL cDNA (1:10 diluted), 1 μL of each primer (10 μM solution), 2 μL LightCycler FastStart DNA Master SYBR Green mix, 3 mM MgCl₂ in a total volume of 20 μL. All templates were amplified with a preincubation for 10 min at 95 °C followed by amplification for 40 cycles: (5 s at 95 °C, 10 s at annealing temperature, 15 s at 72 °C).

In each run, four standards of the gene of interest were included with appropriate dilutions of the DNA, to determine the cDNA concentration in the samples. All RT-PCR amplified a single product as determined by melting curve analysis. To see if the groups differed significantly, a student-*t* test was performed on the log transformed concentrations corrected for the amount of 28S and $p < 0.05$ was considered as significant.

3. RESULTS

3.1. Bacteriological examination and body weight

In all the animals inoculated with *Salmonella enterica* serovar Enteritidis, the *Salmonella* was detected in the feces at all time points analyzed by bacterial platings. In contrast, *S. enterica* serovar Enteritidis was detected in none of the control animals. The number of *S. enterica* serovar Enteritidis found in the liver of chickens from the fast growing (F) and slow growing (S) lines is presented in Figure 1. At days 1 and 5 pi the chickens from line S had more cfu *Salmonella* in the liver compared to line F ($p < 0.05$).

Since Line F was the fast growing chicken line, from day 5 onwards the

Table I. Primers and RT-PCR conditions for different genes.

| Gene	Forward primer	Reverse primer	Annealing temperature
XM_416896 (lysozyme G)	CGGCTTCAGAGAAGATTG	GTACCGTTTGTCAACCTGC	62 °C
XM_416085 (carboxypeptidase M)	ATTCTGGAGAGACAACAAAGTTGCT	TTTGGCTTCCACGATTGCA	58 °C
XM_425603(ORF2)	GTCAGCCTCTTCTCTCGTGTGA	AGTGCCTGACCACCCTTTCA	58 °C
BX930518 (clone ChEST640b17)	GAATCAAGCAACTTCCGTACCAT	AGGTTCCAAGAGCCTGAAAGTTC	59 °C
XM_420282 (DNA segment, Chr 10)	TCTTCCCAGGCTGTGAG	GGTCACCAGCTTGTTCTTC	64 °C
NM_205513(calbindin)	CATGGATGGGAAGGAGC	GCTGCTGGCACCTAAAG	56 °C
DQ_018756 (28S)	TCAACTTTCCCTTACGGTAC	CAAGTCCTTCTGATCGAG	56 °C
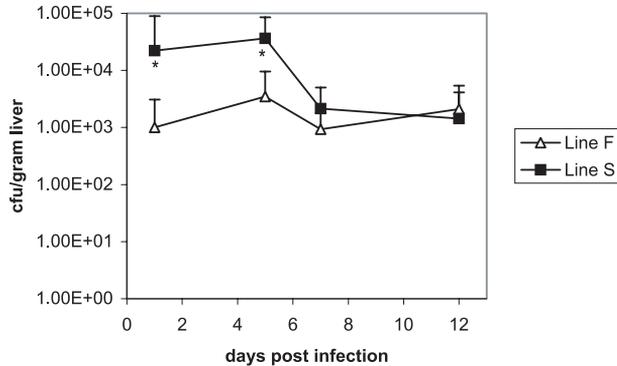
Figure 1. Number of cfu of *S*. *enteritidis* in the liver of chickens from the two chicken lines. Error bars indicate the SEM ($n = 10$). *Infected chickens from line F have significant less cfu *Salmonella* in the liver than chickens from line S ($p < 0.05$).

healthy chickens from this line were heavier than the healthy chickens from line S ($p < 0.001$). The chickens from line S had a weight gain depression due to the *Salmonella* infection ($p < 0.01$) while the chickens from line F had no significant weight gain depression after the *Salmonella* infection (Fig. 2).

3.2. Phagocytic activity and flow cytometric analysis of intestinal cells

The phagocytic activity of the isolated intestinal mononuclear cells was measured with the phagocyte assay and the results are shown in Table II. However, at least 1×10^7 mononuclear cells per chicken were

necessary for this assay and not enough cells were isolated from all jejunums. Therefore, for this assay 3 to 10 animals per group were used. The isolated intestinal mononuclear cells were stained with trypan blue to check the viability of the cells and were analyzed with FACS for the percentage of CD45+ cells. The gated mononuclear CD45+ cells in the jejunum increased with the age of the chickens (Fig. 3). At day 1 pi the *Salmonella* infected chickens from line F had a significant lower percentage CD45+ cells compared to their healthy counterparts ($p < 0.01$).

For the phagocytic activity, only effects within each day can be compared, due to the differences in number of bacteria
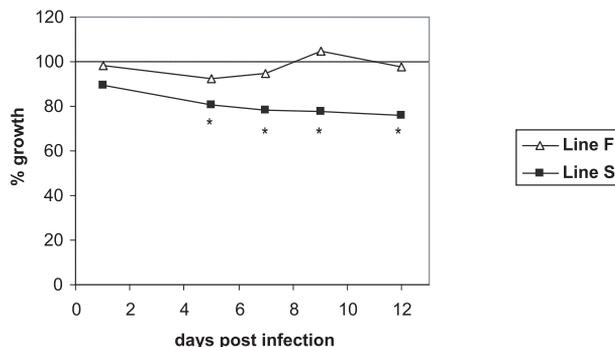
Figure 2. Percentage growth of chickens infected with 10^5 *S. enteritidis* compared to healthy counterparts. *Infected chickens are significantly lighter than age-matched healthy counterparts from the same line ($p < 0.05$).

Table II. Phagocytic activity of mononuclear gut cells of chickens[a].

Line	Day 1		Day 5		Day 7	
	control	infected	control	infected	control	infected
F	9 ($\pm$ 5)[b]	64 ($\pm$ 9)	5 ($\pm$ 1)	5 ($\pm$ 4)	357 ($\pm$ 26)	364 ($\pm$ 32)
S	33 ($\pm$ 10)	48 ($\pm$ 8)	7 ($\pm$ 1)[b]	16 ($\pm$ 4)	378 ($\pm$ 33)	396 ($\pm$ 33)

[a] Total number $\times 10^3$ of internalized bacteria by all cells $\pm$ SEM.
[b] Significant difference between control and *Salmonella* infected chickens ($p < 0.05$).

between the overnight *Salmonella* cultures for the different days pi. At day 1 pi the cells isolated from the *Salmonella* infected chickens from line F had almost 7 times more bacteria internalized compared with the cells from the control chickens ($p < 0.01$). In contrast, in line S no differences between the cells of the control chickens and the *Salmonella* infected chickens were found at day 1 pi (Tab. II). However, at day 5 pi the cells from the *Salmonella* infected chickens from line S internalized two times more bacteria than the cells from the control chickens from the same line ($p < 0.05$). At day 5 pi in line F, no differences in the amount of internalized bacteria between the control and the *Salmonella* infected chickens were found (Tab. II). At day 7 pi no differences in phagocytic activity were found between

cells from the *Salmonella* infected and the control chickens.

3.3. Immunohistochemistry

Frozen jejunum sections from all animals were quantified for CD4 positive T-cells, CD8 positive T-cells and macrophages with immunohistochemistry and we found small but significant differences between the groups (Tab. III). The number of CD4$^+$ T-cells per mm^2 was at day 1 and day 5 pi lower in the *Salmonella* infected chickens from line F compared to the healthy age-matched controls from the same line. There were no clear differences in the location of the CD4$^+$ T-cells, since most cells were located in the lamina propria. For line S no differences in the
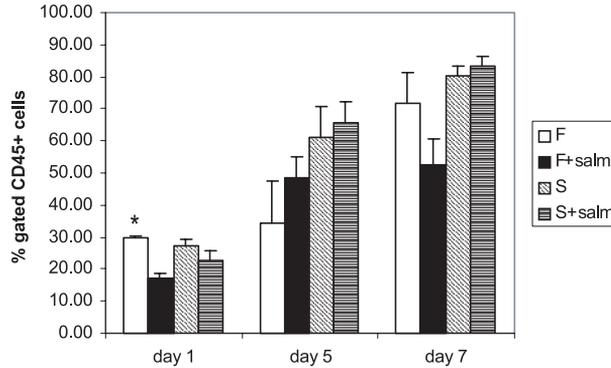
Figure 3. Amount of CD45^{+} cells in the isolated intestinal mononuclear cells as a percentage of the gated intestinal mononuclear cells. F= fast growing chicken line, S = slow growing chicken line, + salm = infected with *Salmonella enteritidis*. Standard bars indicate the SEM (n = 3–10). * Significant difference between cells from control and infected chickens ($p < 0.01$).

Table III. Mean number ($\pm$ SEM) of CD4^{+}, CD8^{+} and macrophages per mm^2.

Line	Day 1		Day 5		Day 7	
	Control	Infected	Control	Infected	Control	Infected
CD4^{+} T-cells						
F	14 ($\pm$ 2)[a]	8 ($\pm$ 1)	45 ($\pm$ 3)[a]	33 ($\pm$ 3)	100 ($\pm$ 4)	113 ($\pm$ 7)
S	12 ($\pm$ 2)	16 ($\pm$ 2)	68 ($\pm$ 5)	54 ($\pm$ 8)	74 ($\pm$ 7)	91 ($\pm$ 5)
CD8^{+} T-cells						
F	38 ($\pm$ 4)	57 ($\pm$ 8)	329 ($\pm$ 27)	300 ($\pm$ 29)	211 ($\pm$ 21)[a]	335 ($\pm$ 24)
S	32 ($\pm$ 6)	26 ($\pm$ 3)	168 ($\pm$ 11)[a]	237 ($\pm$ 24)	193 ($\pm$ 15)[a]	254 ($\pm$ 19)
Macrophages						
F	226 ($\pm$ 14)	241 ($\pm$ 17)	353 ($\pm$ 16)	382 ($\pm$ 19)	451 ($\pm$ 44)	485 ($\pm$ 36)
S	213 ($\pm$ 14)[a]	124 ($\pm$ 8)	391 ($\pm$ 30)	395 ($\pm$ 25)	469 ($\pm$ 25)	457 ($\pm$ 16)

[a] Significant difference between control and *Salmonella* infected chickens ($p < 0.05$).

amount or location of CD4^{+} T-cells was found between the control and the infected chickens. The number of CD8^{+} T-cells was increased at day 7 pi in the infected chickens from line S compared to their controls and at days 5 and 7 pi for the infected chickens from line F.

At day 1 pi, the number of macrophages in the *Salmonella* infected chickens from line S was lower than the healthy chick-ens from line S or the *Salmonella* in-fected chickens from line F. At later time-points, no significant differences between the amounts of macrophages were found.

3.4. Gene expression in the chicken intestine

RNA was isolated from the chicken jejunum day 1 pi to investigate gene

Table IV. Fold changes in mRNA compared with age-matched mock-infected controls 24 h after the *Salmonella* infection.

Accession No.	Gene name	Foldchange (infected/ control)	
		Line F	Line S
Genes regulated in line S after *Salmonella* infection			
XM_416896.1	PREDICTED: *Gallus gallus* similar to lysozyme G	1.0	6.9
CR522945	finished cDNA, clone ChEST753p12	1.3	4.2
XM_418587	PREDICTED: *Gallus gallus* similar to CG3524-PA (LOC420485)	1.5	3.1
DN828701	expressed sequence tag (Chr:2 80621800 -80622343)	–1.7	–27.4
XM_416085.1	PREDICTED: *Gallus gallus* similar to Carboxypeptidase M precursor (LOC417843), mRNA	–2.0	–7.2
XM_425603.1	PREDICTED: *Gallus gallus* similar to ORF2 (LOC428036), mRNA	–2.3	–6.1
XM_423002.1	similar to Rho GTPase-activating protein; brain-specific Rho GTP-ase-activating protein	–1.8	–4.7
BX930518.1	*Gallus gallus* finished cDNA, clone ChEST640b17	–1.0	–3.5
BU457068.1	cDNA clone ChEST200c16	–1.2	–3.3
Genes regulated in line F after *Salmonella* infection			
NM_204192.1	*Gallus gallus* fatty acid binding protein 1, liver	–4.0	–1.1
Genes regulated after a *Salmonella* infection in both chicken lines			
XM_420282.1	PREDICTED: *Gallus gallus* similar to DNA segment, Chr 10, Johns Hopkins University 81 expressed	4.8	19.1
NM_001006208	*Gallus gallus* ras homolog gene family, member T1 (RHOT1)	7.9	15.0
NM_205125.1	*Gallus gallus* dickkopf homolog 3	4.7	14.2
XM_418586	PREDICTED: *Gallus gallus* similar to Fatty acid synthase	3.7	6.0
NM_204933.1	*Gallus gallus* cytidine deaminase (CDD)	2.9	3.6
NM_205513.1	*Gallus gallus* calbindin 1, 28 kDa	–3.2	–3.8

expression responses to *Salmonella*. By comparing the gene expression responses of both chicken lines, we found more differences than similarities 1 day pi. After the *Salmonella* infection three genes were more than threefold upregulated and six genes were more than threefold downregulated in line S, but not in line F (Tab. IV). In line F, liver fatty acid binding protein was downregulated after the infection, whereas

no significant regulation was observed in line S.

In addition to the genes regulated after the *Salmonella* infection in one of the chicken lines, some genes were regulated in both chicken lines. Similar to DNA segment, Chr 10, ras homolog gene family member T1, dickkopf homolog 3, similar to fatty acid synthase and cytidine deaminase were upregulated, whereas calbindin

Table V. Expression differences found with the microarray compared with the q-PCR.

Gene	Line F Ratio *Salmonella*/control[a]		Line S Ratio *Salmonella*/control[a]	
	Microarray	q-PCR	Microarray	q-PCR
XM_416896 (lysozyme G)	1.0	−1.0	6.9	14.0[b]
XM_416085 (carboxipeptidase M)	−2.0	−1.1	−7.2	−3.2[b]
XM_425603 (ORF2)	−2.3	−1.3	−6.1	−2.4[b]
BX930518 (clone ChEST640b17)	−1.0	−1.1	−3.5	−3.0[b]
XM_420282 (DNA segment, Chr 10)	4.8	3.9[b]	19.1	14.1[b]
NM_205513 (calbindin)	−3.2	−2.6[b]	−3.8	−2.8[b]

[a] When the ratio (*Salmonella*/control) is smaller than 1, the ratio −(control/*Salmonella*) is given.
[b] The expression levels of the control and Salmonella infected group from the same chicken line differ significantly (student *t*-test, $p < 0.05$).

was downregulated more than threefold in both chicken lines (Tab. IV).

For lysozyme G, carboxypeptidase M, similar to ORF2, cDNA clone ChEST640b17, similar to DNA segment, Chr 10 and calbindin a quantitative PCR was performed on the individual samples. For all these genes, the up- or downregulation we found with the microarray was confirmed with the RT-PCR. Furthermore, when more than threefold expression differences were detected with the microarray, which was our threshold to call a gene up- or downregulated, the expression levels differed significantly ($p < 0.05$) between the control and the *Salmonella* infected chickens from the same line (Tab. V).

4. DISCUSSION

Salmonella bacteria that reach the intestinal tract can cross the intestinal epithelium after attachment to the mucosa. From there, they can reach the lamina propria, where they replicate or penetrate into deeper tissues. After reaching the blood stream, they infect internal organs, such as the liver and spleen. In our experiment, colonization of the liver started already 1 day

post inoculation, with 40 and 90% of the chickens positive from line F and S respectively. It was unexpected that the chickens from line F had no body weight loss, because it was suggested that fast growing meat-type chickens are more susceptible to *Salmonella* compared with slow growing ones [13]. On the contrary, it has also been reported that meat-type chickens, which grow fast, are more resistant to *Salmonella* compared with laying-type lines [8]. So overall the relation between *Salmonella* susceptibility and growth rate is not unambiguous. Nevertheless the two chicken lines used in this experiment showed a clear difference in outcome of a *Salmonella* infection and it was interesting to further analyse their intestinal responses to the *Salmonella* infection and to compare gene expression between these lines and lines that were used in an earlier study [23].

At day 1 pi, the isolated intestinal mononuclear cells from the *Salmonella* infected chickens from line F had a higher phagocytic activity than the control animals. These differences were not due to differences in the amount of macrophages as determined by immunohistochemistry. Possibly the macrophages of the infected animals are more active.

When the macrophages are more active, less *Salmonella* bacteria are able to survive. Indeed the number of cfu *Salmonella* in the liver of the infected animals from line F were significantly lower than in the infected chickens from line S. Another option is that the isolated cell suspension of the infected animals from line F are of a different composition compared with the cell suspension of the control animals. The percentage of macrophages in the isolated cell population can differ between infected and control animals causing the difference in the number of phagocytosed bacteria. The infected animals had a lower percentage of CD45+ cells (marker for leukocytes) in the isolated cell population, thus other non CD45+ cells were used in our phagocytose assay, because the amount of mononuclear cells used in the assay was equal among the groups. Thus, either the macrophages are more active or other cells are responsible for a better phagocytic activity in the cell population isolated from the *Salmonella* infected animals.

At day 5 pi, differences in the phagocytic activity between the intestinal mononuclear cells between the infected and the control chickens were found for line S instead of line F as for day 1 pi. This was also not caused by differences in the amount of macrophages. Here no differences in the amount of CD45+ cells in the isolated cell populations were found. At day 7 no differences in phagocytic activity of the intestinal mononuclear cells were found, which is in agreement with an earlier study [12]. Unfortunately in that study, no earlier timepoints than day 7 pi were investigated. So we found at early timepoints post infection differences in the phagocytic activity of the mononuclear cells from the intestine of control and *Salmonella* infected animals, but the causes of these differences are not known.

The number of CD4+ was decreased in line F in response to the *Salmonella* infection, but not in line S. This is surprising because in most studies an increase in the amount of CD4+ T-cells in the caeca was found after a *Salmonella* infection [1, 6]. Also in mice the number of CD4+ T-cells increased in the gut following *Salmonella* challenge [2, 15]. Furthermore in the ovary and oviduct of laying hens, increased numbers of CD4+ T-cells were reported after a *Salmonella* infection [4, 25, 26]. However, for the early host response CD4+ or CD8+ T-cells are probably not required [14]. In addition here young chickens were investigated where the intestinal immune system is immature, which could be a reason that no increase in CD4+ T-cells was found. For CD8+ cells, we found an increase after the *Salmonella* infection in both chicken lines, but faster in time post infection in line F. An increase in the amount of CD8+ cells was also found in the caeca after a *Salmonella* infection [1, 6], but also decreases in the amount of CD8+ cells in the caeca are reported [18]. In the oviduct of laying hens, the numbers of CD8+ cells were increased after a *Salmonella* infection [4, 25, 26]. Our and other results suggest that differences in influx of T-cell subpopulations after a *Salmonella* infection are dependent on the location in the digestive tract, infection dose, time post infection, age at the time of infection and genetic background of the chickens.

The gene expression as measured with the microarray and validated with quantitative PCR in the jejunum at day 1 pi in these two chicken lines was partial in correspondence with our earlier study [23]. Besides differences in gene expression responses between the two chicken lines, we again saw upregulation in both lines of cytidine deaminase, similar to fatty acid synthase, dickkopf homolog 3 and similar to DNA segment, Chr 10, Johns Hopkins University 81 expressed in response to the *Salmonella* infection. It is noteworthy that all these genes were more upregulated in line S, which had higher growth retardation and more cfu in the liver after the

Salmonella infection than line F. However, in earlier experiments no relation was found with severity of the systemic infection and level of upregulation [23]. So these four genes are upregulated during a *Salmonella* infection, irrespective of the severity of the systemic infection and the growth retardation.

Some of the downregulated genes due to the *Salmonella* infection in line S might be related to decreased amounts of macrophages, as shown by in situ hybridization. One of the downregulated genes is carboxypeptidase M, a macrophage differentiation marker [17]. Also similar to ORF2 (LOC428036) was downregulated, whereas this gene was shown before to be upregulated in avian macrophages after phagocytosis of *Escherichia coli* [7], so lower expression levels of this genes might indicate a decrease in the amount of macrophages.

This is the first report about changes in T-cell subpopulations and macrophages in the chicken jejunum in response to a *Salmonella* infection in one day old broilers and these changes were different between the two lines used. We also found gene expression differences between the two chicken lines, so the genetic background of the chicken is important for their responses to *Salmonella* infection.

REFERENCES

[1] Asheg A., Levkut M., Revajova V., Sevcikova Z., Kolodzieyski L., Pistl J., T lymphocyte subpopulations and B lymphocyte cells in caecum and spleen of chicks infected with *Salmonella enteritidis*, Acta Histochem. (2002) 104:435−439.

[2] Bao S., Beagley K.W., France M.P., Shen J., Husband A.J., Interferon-gamma plays a critical role in intestinal immunity against *Salmonella typhimurium* infection, Immunology (2000) 99:464−472.

[3] Barrow P.A., Huggins M.B., Lovell M.A., Simpson J.M., Observations on the pathogenesis of experimental *Salmonella ty-*

[4] Barua A., Yoshimura Y., Ovarian cell-mediated immune response to *Salmonella enteritidis* infection in laying hens (*Gallus domesticus*), Poult. Sci. (2004) 83:997−1002.

[5] Beal R.K., Powers C., Wigley P., Barrow P.A., Kaiser P., Smith A.L., A strong antigen-specific T-cell response is associated with age and genetically dependent resistance to avian enteric salmonellosis, Infect. Immun. (2005) 73:7509−7516.

[6] Berndt A., Methner U., Gamma/delta T cell response of chickens after oral administration of attenuated and non-attenuated *Salmonella typhimurium* strains, Vet. Immunol. Immunopathol. (2001) 78:143−161.

[7] Bliss T.W., Dohms J.E., Emara M.G., Keeler C.L. Jr., Gene expression profiling of avian macrophage activation, Vet. Immunol. Immunopathol. (2005) 105:289−299.

[8] Guillot J.F., Beaumont C., Bellatif F., Mouline C., Lantier F., Colin P., Protais J., Comparison of resistance of various poultry lines to infection by *Salmonella enteritidis*, Vet. Res. (1995) 26:81−86.

[9] Henderson S.C., Bounous D.I., Lee M.D., Early events in the pathogenesis of avian salmonellosis, Infect. Immun. (1999) 67:3580−3586.

[10] Hughes E.A., Galan J.E., Immune response to *Salmonella*: location, location, location? Immunity (2002) 16:325−328.

[11] Jeurissen S.H., Janse E.M., Koch G., de Boer G.F., The monoclonal antibody CVI-ChNL-68.1 recognizes cells of the monocyte-macrophage lineage in chickens, Dev. Comp. Immunol. (1988) 12:855−864.

[12] Kramer J., Visscher A.H., Wagenaar J.A., Boonstra Blom A.G., Jeurissen S.H., Characterization of the innate and adaptive immunity to *Salmonella enteritidis* PT1 infection in four broiler lines, Vet. Immunol. Immunopathol. (2001) 79:219−233.

[13] Kramer J., Visscher A.H., Wagenaar J.A., Cornelissen J.B., Jeurissen S.H., Comparison of natural resistance in seven genetic groups of meat-type chicken, Br. Poult. Sci. (2003) 44:577−585.

[14] Mastroeni P., Chabalgoity J.A., Dunstan S.J., Maskell D.J., Dougan G., *Salmonella*: immune responses and vaccines, Vet. J. (2001) 161:132−164.

phimurium infection in chickens, Res. Vet. Sci. (1987) 42:194−199.

[15] Mittrucker H.W., Kaufmann S.H., Immune response to infection with *Salmonella typhimurium* in mice, J. Leukoc. Biol. (2000) 67:457−463.

[16] Rabsch W., Tschape H., Baumler A.J., Non-typhoidal salmonellosis: emerging problems, Microbes Infect. (2001) 3:237−247.

[17] Rehli M., Krause S.W., Kreutz M., Andreesen R., Carboxypeptidase M is identical to the MAX.1 antigen and its expression is associated with monocyte to macrophage differentiation, J. Biol. Chem. (1995) 270:15644−15649.

[18] Sasai K., Aita M., Lillehoj H.S., Miyamoto T., Fukata T., Baba E., Dynamics of lymphocyte subpopulation changes in the caecal tonsils of chickens infected with *Salmonella enteritidis*, Vet. Microbiol. (2000) 74:345−351.

[19] Suzuki S., Pathogenicity of *Salmonella enteritidis* in poultry, Int. J. Food Microbiol. (1994) 21:89−105.

[20] Tusher V.G., Tibshirani R., Chu G., Significance analysis of microarrays applied to the ionizing radiation response, Proc. Natl. Acad. Sci. USA (2001) 98:5116−5121.

[21] Van Hemert S., Ebbelaar B.H., Smits M.A., Rebel J.M.J., Generation of EST and microarray resources for functional genomic studies on chicken intestinal health, Anim. Biotechnol. (2003) 14:133−143.

[22] Van Hemert S., Hoekman A.J., Smits M.A., Rebel J.M.J., Differences in intestinal gene expression profiles in broiler lines varying in susceptibility to malabsorption syndrome, Poult. Sci. (2004) 83:1675−1682.

[23] Van Hemert S., Hoekman A.J., Smits M.A., Rebel J.M.J., Early host gene expression responses to a *Salmonella* infection in the intestine of chickens with different genetic background examined with cDNA and oligonucleotide microarrays, Comp. Biochem. Physiol. D (2006) 1:292−299.

[24] Van Immerseel F., De Buck J., De Smet I., Mast J., Haesebrouck F., Ducatelle R., Dynamics of immune cell infiltration in the caecal lamina propria of chickens after neonatal infection with a *Salmonella enteritidis* strain, Dev. Comp. Immunol. (2002) 26:355−364.

[25] Withanage G.S., Sasai K., Fukata T., Miyamoto T., Baba E., Lillehoj H.S., T lymphocytes, B lymphocytes, and macrophages in the ovaries and oviducts of laying hens experimentally infected with *Salmonella enteritidis*, Vet. Immunol. Immunopathol. (1998) 66:173−184.

[26] Withanage G.S., Sasai K., Fukata T., Miyamoto T., Lillehoj H.S., Baba E., Increased lymphocyte subpopulations and macrophages in the ovaries and oviducts of laying hens infected with *Salmonella enterica* serovar Enteritidis, Avian Pathol. (2003) 32:583−590.

[27] Withanage G.S., Wigley P., Kaiser P., Mastroeni P., Brooks H., Powers C., Beal R., Barrow P., Maskell D., McConnell I., Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge, Infect. Immun. (2005) 73:5173−5182.