Curcuma and *Scutellaria* plant extracts protect chickens against inflammation and *Salmonella* Enteritidis infection

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ABSTRACT After a ban on the use of antibiotics as growth promoters in farm animals in the European Union in 2006, an interest in alternative products with antibacterial or anti-inflammatory properties has increased. In this study, we therefore tested the effects of extracts from *Curcuma longa* and *Scutellaria baicalensis* used as feed additives against cecal inflammation induced by heat stress or *Salmonella* Enteritidis (*S.* Enteritidis) infection in chickens. *Curcuma* extract alone was not enough to decrease gut inflammation induced by heat stress. However, a mixture of *Curcuma* and *Scutellaria* extracts used as feed additives decreased gut inflammation induced by heat or *S.* Enteritidis, decreased *S.* Enteritidis counts in the cecum but was of no negative effect on BW or humoral immune response. Using next-generation sequencing of 16S rRNA we found out that supplementation of feed with the 2 plant extracts had no effect on microbiota diversity. However, if the plant extract supplementation was provided to the chickens infected with *S.* Enteritidis, *Faecalibacterium*, and *Lactobacillus*, both bacterial genera with known positive effects on gut health were positively selected. The supplementation of chicken feed with extracts from *Curcuma* and *Scutelleria* thus may be used in poultry production to effectively decrease gut inflammation and increase chicken performance.

Key words: chicken, inflammation, feed, *Scutellaria*, *Curcuma*, *Salmonella*, flavonoid

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INTRODUCTION

After a ban on the use of antibiotics as growth promoters in farm animals in the European Union in 2006 (Castanon, 2007), an interest in alternative products with antibacterial or anti-inflammatory activities has increased. Such products are usually sought among secondary plant metabolites, e.g., flavonoids (Kamboh and Zhu, 2014; Siler et al., 2014). The positive effect of flavonoids on animal health is associated with their antioxidant activity which, at a cellular level, allows the maintaining of normal cell functions even in the presence of oxidative stress, e.g., by preventing membrane lipid oxidation (Hanasaki et al., 1994; Cook and Samman, 1996). However, some researchers argue that the concentration of flavonoids tested as antioxidants in vitro are hardly achievable in vivo, which indicates that other activities such as metal chelation or protein aggregation may be relevant for beneficial action of flavonoids in vivo (Surai, 2014). Plant extracts and flavonoids also affect leukocyte adhesion to the endothelium (Friesenecker et al., 1995) or a release of arachidonic acid (Ferrandiz and Alcaraz, 1991; Laughton et al., 1991; Korbecki et al., 2013), thus contributing to anti-inflammatory effects.

Salmonella enterica belongs among the most common zoonotic agents responsible for gastrointestinal disorders of bacterial origin in humans. Most cases of salmonellosis are associated with consumption of raw or undercooked poultry meat and eggs (Cox et al., 2011; Mughini-Gras et al., 2014). It is therefore assumed that reduction in *Salmonella* prevalence in poultry will result in a decrease in incidence of human salmonellosis. There are several measures which can reduce *Salmonella* prevalence in poultry flocks. Chickens can be protected against *Salmonella* infection by vaccination (Matulova et al., 2012a). It is also possible to improve zoohygienic conditions and biosecurity in poultry production (Fraser et al., 2010). Additional interventions include feed modification, *e.g.* its acidification which may lead to direct *Salmonella* inactivation or modification of conditions in chicken gut which decreases *Salmonella* colonization (Heres et al., 2004). Alternative to feed acidification is its supplementation with different plant extracts with antibacterial or anti-inflammatory effects.

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Plant extracts as feed supplements have been repeatedly tested in chickens. Hesperidin, quercetin or soybean isoflavones tested as feed additives were shown to increase the total antioxidant capacity in different tissues (Jiang et al., 2007; Rupasinghe et al., 2010; Goliomytis et al., 2014). Genistein and hesperidin improved feed intake and decreased inflammatory markers in heat stressed broilers (Kamboh et al., 2013). Feed supplementation with *Curcuma longa* extracts also suppressed the severity of coccidiosis and *Eimeria* multiplication in chickens (Kim et al., 2013). *Curcuma* in combination with extracts from additional plants proved to decrease clinical signs of infection with *Clostridium perfringens* or *Salmonella* Gallinarum (Jung et al., 2010; Lee et al., 2013).

Because of the previous reports on the antibacterial and anti-inflammatory action of extracts from *Curcuma*, we tested *Curcuma longa* extracts, either alone or in combination with *Scutellaria baicalensis* extract as chicken feed additive. The effect of *Scutellaria baicalensis* used as feed additive on gut immune response and microbiota of chickens have not been tested so far, however, it has been reported that wogonin, a key flavonoid present in *Scutellaria baicalensis*, inhibited lipopolysaccharide-induced angiogenesis in vitro (Chen et al., 2009). The aim of this study was therefore to test whether *Curcuma* and *Scutellaria* extracts may 1) reduce gut inflammation in chickens, 2) reduce *Salmonella enterica* serovar Enteritidis (**S. Enteritidis**) infection, and 3) affect gut microbiota composition. The inflammation was induced by 2 different stimuli. First we tested gut inflammation induced by heat stress. Although we observed a positive effect of plant extract supplementation, due to the low inductive effect of heat stress on gut inflammation, the differences in heat stressed chickens fed on a diet with or without plant extracts were quite low. In the repeated experiment we therefore tested the plant extract supplementation in chickens infected with *S.* Enteritidis. Since we found out that a mixture of *Curcuma* and *Scutellaria* extracts decreased *S.* Enteritidis colonization and gut inflammation, finally we analyzed what other microbiota members could be affected by plant extract supplementation using next generation sequencing of V3 and V4 variable region of 16S rRNA genes.

MATERIALS AND METHODS

Ethical Statement

The care and use of chickens in the heat stress study were performed in compliance with the guidelines of the French Ministry of Agriculture and Fisheries for animal research, and was approved by a local ethical committee (Pays de la Loire, France).

S. Enteritidis infection was performed in accordance with current Czech legislation (Animal Protection and Welfare Act No. 246/1992 Coll. of the Government of the Czech Republic). The specific studies were approved by the Ethics Committee of the Veterinary Research Institute followed by the Committee for Animal Welfare of the Ministry of Agriculture of the Czech Republic (Permit Number MZe1449).

Study 1 – Heat Stress

Sixteen newly hatched chicks of the Ross PM3 chicken line were divided into 3 groups with 8 chickens in Group 1 and 4 chickens in Groups 2 and 3. Chickens in Group 1 received the standard diet. Chickens in Group 2 received standard diet supplemented with liposoluble phenolic acid extract from *Curcuma* and chickens in Group 3 received the standard diet supplemented with the extract from *Curcuma* and hydrosoluble flavonoids extract from *Scutellaria,* each forming 0.2% complete feed. The supplemented diets were provided from d 9 life. Chickens were kept at 22°C. Four chickens in Group 1 were sacrificed at the age of 22 d. From d 22, the remaining chickens were subjected to heat stress at 35°C for 2 d and sacrificed immediately after, i.e., when aged 24 d. During necropsy, pieces of cecal tissue were collected into RNALater and the samples were stored at −80°C prior RNA purification.

Study 2 – S. Enteritidis Infection

Fourty-eight newly hatched ISA Brown males of the egg laying chicken line were divided into 2 groups. Group 1 received a standard diet and Group 2 received a standard feed supplemented with liposoluble phenolic acid extract from *Curcuma* and hydrosoluble extract from *Scutellaria*. The supplemented diets were provided from the d 1 life. Seven d later, half of the chickens in each group were infected with 1×10^7 cfu *S.* Enteritidis 147 spontaneously resistant to nalidixic acid (Methner et al., 2004). The remaining chickens were used as non-infected controls. Four and 14 d postinfection, 6 chickens from each group were sacrificed under chloroform anesthesia. Cecal tissues were collected into RNALater and the samples were stored at −80°C. Cecal contents were collected and frozen at −20°C until DNA purification. To enumerate *S.* Enteritidis, samples of 0.5 g spleen, liver, and cecum were collected as well.

Enumeration of S. Enteritidis

Samples were homogenized in peptone water, 10-fold serially diluted, and plated on xylose lysine deoxycholate agar plates (HiMedia) supplemented with 20 μg/mL nalidixic acid. Samples that were negative after plating were subjected to a pre-enrichment in buffered peptone water and enrichment in modified semi-solid Rappaport–Vassiliadis medium (Oxoid) for qualitative determination of *Salmonella*. *Salmonella* counts positive after direct plating were logarithmically

Table 1. Primers used in real-time PCR

Gene	Primer Sequence (5′ to 3′) Forward	Reverse	GenBank Accession	Reference
AVD[1]	CCTTTGGCTTCACTGTCAAT	GCGAGTGAAGATGTTGATGC	NM_205320.1	(Matulova et al., 2012b)
AH221	CTCTGCTCCTCGGCTGTG	TCCTTCCCTTTCTTGGTCAC	XM_415780.4	(Matulova et al., 2012b)
C3	AACGCCCACACCTACAACAT	ACGTGGTACTGAGCCAGAGC	NM_205405.1	(Matulova et al., 2013)
ExFABP	GGAACTACACGGATGAGATGGT	TGGCACATTAGTCTTGCTTTGT	NM_205422.1	(Matulova et al., 2012b)
HPX	CGTGATCTCCGTGACTACTTCA	GCCACTGTCATCAGAGGTGA	XM_417267.4	(Matulova et al., 2013)
IFNγ	GCCGCACATCAAACACATATCT	TGAGACTGGCTCCTTTTCCTT	NM_205149.1	(Berndt et al., 2007)
IgA	AGGATGGTCCTCCAGAAGGT	CAACAACAGTCGGACAGCAC	AF190134.1	(Matulova et al., 2012b)
IgY	CTATGGGGCAGGAGTGAGAA	GACCACCTGACCCACAGATT	AB029077.1	(Matulova et al., 2012b)
IL1β	GAAGTGCTTCGTGCTGGAGT	ACTGGCATCTGCCCAGTTC	NM_204524.1	(Crhanova et al., 2011)
IL8L2	CAAGCCAAACACTCCTAACCAT	AGCTCATTCCCCATCTTTACC	NM_205498.1	(Crhanova et al., 2011)
IL17	TATCAGCAAACGCTCACTGG	AGTTCACGCACCTGGAATG	NM_204460.1	(Crhanova et al., 2011)
IL22	CAGGAATCGCACCTACACCT	TCATGTAGCAGCGGTTGTTC	NM_001199614.1	(Crhanova et al., 2011)
iNOS	GAACAGCCAGCTCATCCGATA	CCCAAGCTCAATGCACAACTT	NM_204961.1	(Berndt et al., 2007)
IRG1	ACCGAGGTCTGCCAGAAAGT	TCGTCGAAATCCATTGAGTG	NM_001030821.1	(Matulova et al., 2012b)
PGDS	CATTCCTGTGCAAGCTGACTT	CTGTTCCTCTTCTCGCACTGTT	NM_204259.1	(Matulova et al., 2013)
SAA	TAGTTTGCCTCACGCATGTC	GCTTCGTGTTGCTCTCCATT	XM_004941433.1	(Matulova et al., 2012b)
STAT1	CCGATACACATGGCAATGATAA	TGCATCAAGCTCCTTCTGTTTA	NM_001012914.1	(Matulova et al., 2013)
STAT3	TAGTGCTGCTCCGTATCTGAAG	CAGGTCAATGGTATTGCTGAAG	NM_001030931.1	(Matulova et al., 2013)
GAPDH[2]	CCTGCATCTGCCCATTT	GGCACGCCATCACTATC	NM_204305.1	(De Boever et al., 2008)
TBP[2]	TAGCCCGATGATGCCGTAT	GTTCCCTGTGTCGCTTGC	NM_205103.1	(Li et al., 2005)
UB[2]	CTTGCCAGCAAAGATCAACCTT	GGGATGCAGATCTTCGTGAAA	XM_004946671.1	(De Boever et al., 2008)

[1]*AVD* = Avidin; *AH221* (*CCLi10*) = Chemokine AH221, CC chemokine; *C3* = Complement component 3; *ExFABP* (p20k; *LCN8*) = Extracellular fatty-acid binding protein, lipocalin 8; *GAPDH* = Glyceraldehyde 3-phosphate dehydrogenase; *HPX* = Hemopexin; *IFNγ* = Interferon gamma; *IgA* = Immunoglobulin A, constant part; *IgY* = Immunoglobulin Y, constant part; *IL1β* = Interleukin 1 beta; *IL8L2* (*CXCLi2*) = Interleukin 8-like 2, CXC chemokine; *IL17* = Interleukin 17F; *IL22* = Interleukin 22; *iNOS* = Inducible NO synthase (*NOS2*); *IRG1* = Immune responsive gene 1 (mouse homolog); *PGDS* = Prostaglandin D2 synthase; *SAA* = Serum amyloid A; *STAT1* = Signal transducer and activator 1; *STAT3* = Signal transducer and activator 3; *TBP* = TATA box binding protein; *UB* = Polyubiquitin C-like.

[2]House-keeping genes.

transformed. Samples that were positive only after enrichment were assigned a value of 1 and negative samples were assigned a value of 0.

Quantitative Reverse-Transcriptase PCR

Approximately 25 mg cecal wall was transferred into 1 mL TRI Reagent (Molecular Research Center) and homogenized using zirconia silica beads (BioSpec Products) in a MagNALyser (Roche). Fifty μL bromoanisole (Molecular Research Center) was added to the homogenate, the samples were vigorously shaken for 10 s, and centrifuged at 4°C for 15 min at 12,000 × *g*. The upper aqueous phase (500 μL) was collected and mixed with an equal volume of 70% ethanol. This mixture was applied onto RNeasy purification columns and washing and RNA elution was performed exactly as suggested by the manufacturer (Qiagen). The concentration and purity of the RNA was determined spectrophotometrically (Nanodrop, Thermo Fisher Scientific). One μg RNA was immediately reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and oligo (dT) primers. cDNA was diluted 10× with sterile water and stored at −20°C until real-time PCR. The expression of 16 genes was determined by real-time PCR. real time PCR was performed in 3-μL volumes in 384-well microplates using QuantiTect SYBR Green PCR Master Mix (Qiagen) and a Nanodrop pipetting station (Inovadyne) for PCR mix dispensing. PCR and signal detection were performed using a LightCycler II (Roche) with an initial denaturation at 95°C for 15 min followed by 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s. Each sample was subjected to real-time PCR in duplicate and the mean values of the duplicates were used for subsequent analysis. The Ct values of the genes of interest were normalized (ΔCt) to an average Ct value of three house-keeping genes, glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***), ubiquitin (***UB***), and TATA box binding protein (***TBP***), and the relative expression of each gene of interest was calculated as $2^{-\Delta Ct}$. Primers are listed in Table 1.

Sequencing V3/V4 Region of 16S rRNA Genes

Cecal content samples were homogenized using zirconia silica beads (BioSpec Products) in a MagNALyzer (Roche Diagnostics). Following homogenization, the DNA was extracted using the QIAamp DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen). The DNA concentration and quality was determined spectrophotometrically and the DNA was stored at −20°C until use. DNA samples from the cecal contents of 47 chickens (the cecum of one *S.* Enteritidis infected chicken 14 d postinfection was empty and we were unable to collect enough cecal content) were diluted to the same concentration of 5 ng/μL and

used as a template in PCR with forward primer 5′-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*-MID-GT-CCTACGGGNGGCWGCAG-3′ and reverse primer 5′-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*-MID-GT-GACTACHVGGGTATCTAATCC-3′. The sequences in italics served for index and adapter ligation whereas underlined sequences allowed for the amplification over V3/V4 region of 16S rRNA genes. MIDs represent different sequences of 5, 6, 9, or 12 base pairs in length and these were designed to inner differentiation of samples in groups. PCR amplification and clean up were performed using KAPA Taq HotStart PCR Kit (Kapa Biosystems) following the protocol for 16S metagenomic sequencing library preparation recommended by Illumina. In the next step the DNA concentration was determined fluorimeterically and the DNA was diluted to the same concentration 100 ng/μL. Groups of 14 PCR products with different molecular identifier sequences were pooled and indexed with a Nextera XT Index Kit following the manufacturer's instructions (Illumina). Prior to the sequencing, concentration of differently indexed samples was determined by a KAPA Library Quantification Complete kit (Kapa Biosystems). All indexed samples were diluted to 4 ng/μL and 20% of phiX DNA was added. Sequencing was performed using MiSeq Reagent Kit v3 and MiSEQ 2000 apparatus according to the manufacturer's instructions (Illumina). The raw sequence reads have been deposited in the National Center for Biotechnology Information Short Read Archive under Accession Number PRJNA280159.

The fastq files generated as an Illumina sequencing output were uploaded into Qiime software. Reverse reads from pair end sequencing were shortened to length 250 base pairs and pair ends were joined. Quality trimming criteria were set to a value 19 and no mismatch in the molecular identifier sequences. In the next step, chimeric sequences were predicted and excluded from the analysis. The resulting sequences were then classified with RDP Seqmatch with an operational taxonomic units discrimination level set up to 97%. The following step included diversity analyses (Chao1 richness, evenness estimation, and Shannon index) on operational diversity analysis clusters, UniFrac analysis, and principal coordinate analysis.

Statistical Analysis

Sequencing data were analyzed using Qiime v.1.8.0 software. ANOVA followed by Tukey's multiple comparison test or Kruskal–Wallis test followed by Dunn's multiple comparison test have been used for the comparison of performance of different chicken groups as indicated in the text. Comparison with P values equal or lower than 0.05 were considered significantly different.

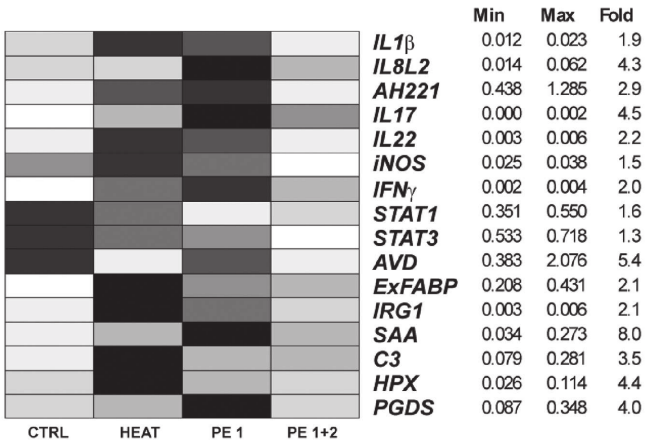

Figure 1. Expression of cytokines and genes coding for acute phase proteins in the cecum of chickens subjected to 2-day-long heat stress. Median values were used to present expression by a heat map; darker shading indicates higher expression level. No statistical differences were found between groups according to non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison test, most likely due to low induction of gut inflammation by heat stress. CTRL = Expression in the chickens fed with standard feed without heat stress; HEAT = Expression in the chickens fed with standard feed subjected to heat stress; PE1 = Expression in the chickens subjected to heat stress fed with feed containing plant extract from *Curcuma* only; PE1+2 = Expression in the chickens subjected to heat stress fed with feed with both *Curcuma* and *Scutellaria* extracts.

RESULTS

Effect of Plant Extract Supplementation on the Expression of Inflammatory Markers in the Cecum of Chickens Exposed to Heat Stress

Since heat stress may induce gut inflammation in chickens (Quinteiro-Filho et al., 2012; Prakasam et al., 2013), first we tested anti-inflammatory activity of plant extracts in the 22-day-old broiler chickens subjected to heat stress for 2 d. Chickens responded to the heat stress with an increased expression of *IL1β*, *IL22*, *iNOS*, *ExFABP*, *IRG1*, *C3*, and *HPX* in the cecum (Figure 1). Feed supplementation with only *Curcuma* extract did not affect chicken response to heat stress. However, feed supplementation with both *Curcuma* and *Scutellaria* extracts caused such chickens to exhibit a similar expression profile of inflammatory markers as the heat nonexposed control chickens (Figure 1).

Effect of Plant Extracts on Chicken Resistance to S. Enteritidis Infection

Since the heat induced expression of inflammatory markers was not numerically high (maximal inductions were around a factor of 5; see Figure 1), in the next experiment we tested the anti-inflammatory effect of the plant extracts against inflammation induced by *S.* Enteritidis infection. In the experiment with *S.* Enteritidis infection we tested only the feed supplemented with both plant extracts. *S.* Enteritidis counts in the

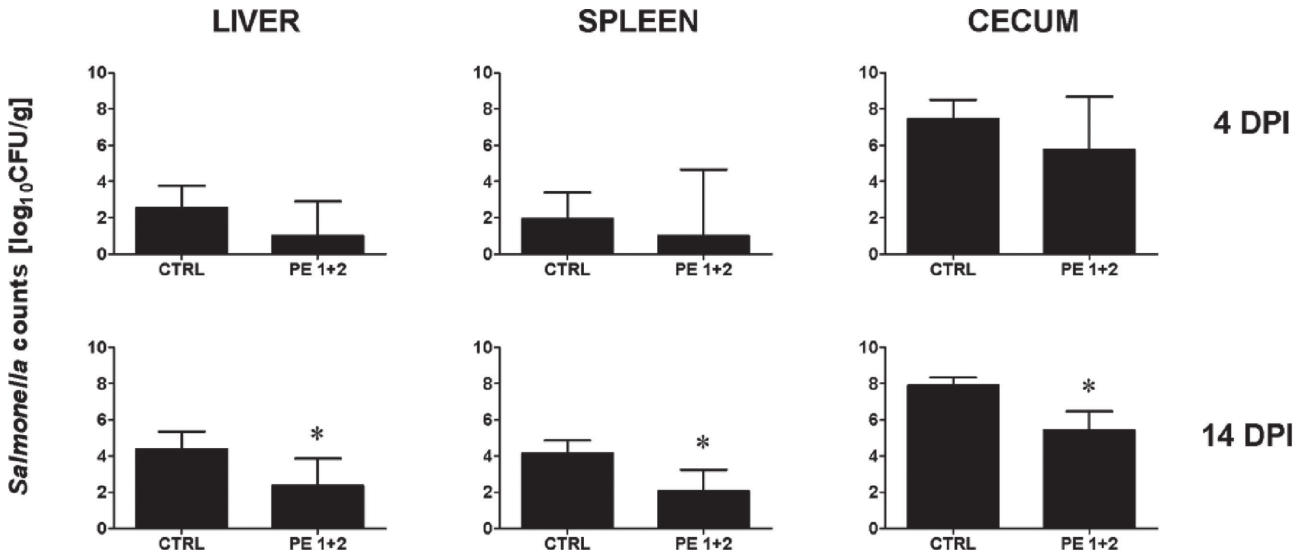

Figure 2. *Salmonella* counts in chicken organs 4 and 14 d postinfection (DPI). Data are presented as mean ± SD. CTRL = Chickens fed the control diet; PE1+2 = Chickens fed the control diet supplemented with a mixture of *Curcuma* and *Scutellaria* plant extracts. Asterisks indicate statistically significant differences at $P < 0.05$ according to ANOVA followed by Tukey's multiple comparison test.

liver, spleen, and cecum of chickens fed with feed supplemented with *Curcuma* and *Scutellaria* extracts were numerically lower than in chickens fed with the control diet, both 4 and 14 d postinfection. However, the difference reached statistical significance only 14 d postinfection (Figure 2).

BW of Chickens Fed with a Diet Supplemented with Plant Extract

To exclude any negative effect of plant extract supplementation on growth, the BW of all the chickens was determined at the end of the study. Weight of the chickens fed with plant extract supplementation did not significantly differ from the weight of the chickens in the control group, irrespective of age or infection with *S.* Enteritidis (Figure 3). Feed supplemented with plant extracts therefore did not affect the growth of the chickens.

Effect of Plant Extracts on the Expression of Chicken Cytokines and Acute Phase Proteins after S. Enteritidis Infection

Differences in *S.* Enteritidis counts (Figure 2) indicated a protective effect of plant extract supplementation against the infection. Next we therefore verified this observation by determining the expression of genes known to be induced in the chicken cecum after *S.* Enteritidis infection. *S.* Enteritidis infection significantly induced expression of all tested genes 4 d postinfection (Figure 4). Except for *IL1β*, *IRG1*, *HPX*, and *PGDS*, the rest of the genes remained significantly induced also at 14 d postinfection although the differences between infected and noninfected chickens were not numerically as pronounced as at 4 d postinfection. However, none of the genes became significantly induced by *S.* Enteritidis in the chickens provided the plant extract supplemented diet in comparison to the noninfected chickens

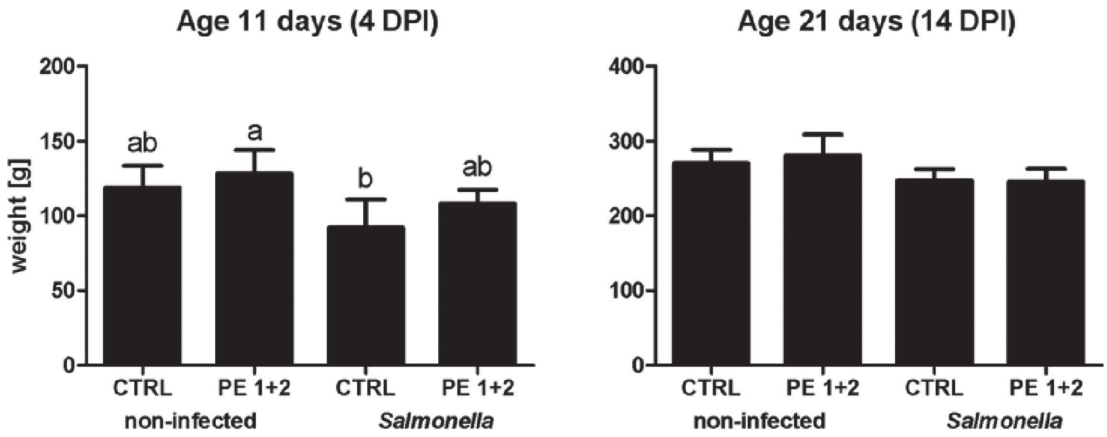

Figure 3. Weight of ISA Brown chickens fed with feed supplemented with plant extracts. Data are presented as mean ± SD. CTRL = Chickens fed control diet; PE1+2 = Chickens fed control diet supplemented with a mixture of *Curcuma* and *Scutellaria* plant extracts. Different letters represent statistically significant differences at $P < 0.05$ according to Kruskal–Wallis followed by Dunn's multiple comparison test.

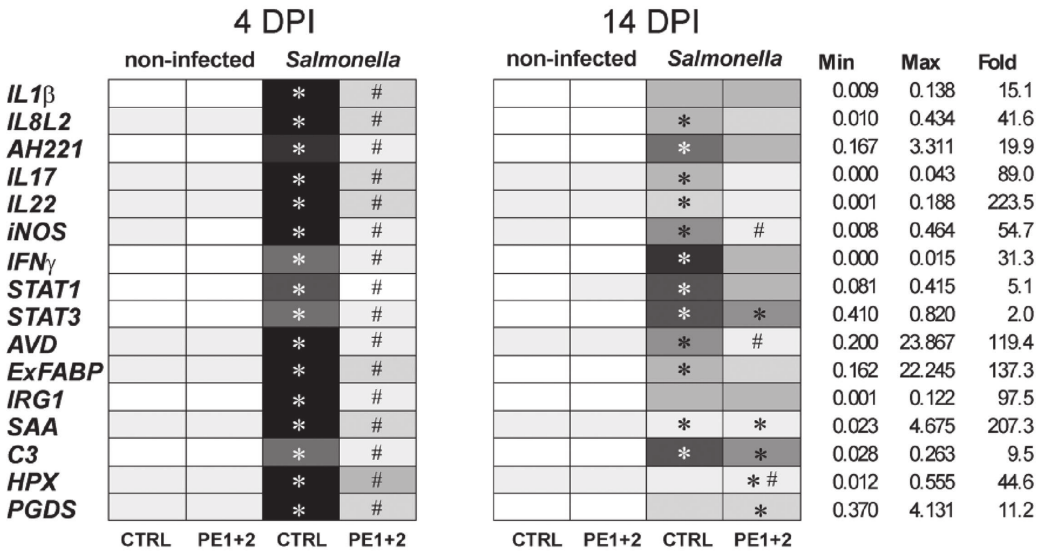

Figure 4. Expression of cytokines and acute phase genes in chickens after *S.* Enteritidis infection. CTRL = Chickens fed control diet; PE1+2 = Chickens fed control diet supplemented with a mixture of *Curcuma* and *Scutellaria* plant extracts. Darker shading indicates higher expression level. Asterisks indicate statistical differences between *S.* Enteritidis infected chickens fed a nonsupplemented diet and the noninfected control group according to ANOVA followed by Tukey's multiple comparison test. Hashes indicate significant differences between *S.* Enteritidis infected chickens fed diets with or without plant extract supplementation. Note the fold difference between minimal and maximal values recorded, especially in comparison with the same range after the heat stress in Figure 1.

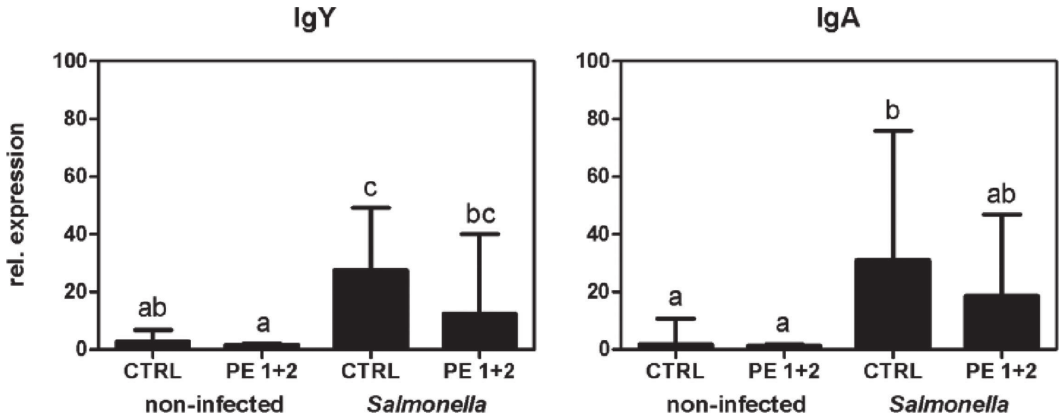

Figure 5. Expression of constant regions of heavy chains of IgY and IgA after *Salmonella* infection. Data are presented as mean ± SD at 14 days post infection. CTRL = Chickens a fed control diet; PE1+2 = Chickens fed a diet supplemented with a mixture of *Curcuma* and *Scutellaria* plant extracts. Different letters represent statistically significant differences at $P < 0.05$ according to Kruskal–Wallis followed by Dunn's multiple comparison test (n = 6, except for *Salmonella*-infected CTRL 14 d postinfection, where n = 5).

at 4 d postinfection and the similar trend in chicken response to *S.* Enteritidis infection and plant extract supplementation was recorded also at 14 d postinfection (Figure 4). Supplementation of feed with *Curcuma* and *Scutellaria* plant extracts therefore protected chickens from inflammatory response to *S.* Enteritidis infection.

Effect of Plant Extracts on Chicken Humoral Response to S. Enteritidis Infection

The difference in inflammatory response in *S.* Enteritidis infected chickens fed a diet with or without plant extracts finally prompted us to test whether the plant extract could interfere with humoral response. Since we did not collect sera from the chickens, instead of determing antibody levels in serum, we determined the amount of IgY and IgA transcripts using cDNA from chicken ceca. There were no differences in the level of IgY and IgA in the cecum 4 d postinfection among the group of chickens, irrespective of infection or feed formula (data not shown). However, at 14 d postinfection, chickens infected with *S.* Enteritidis expressed significantly higher levels of IgY and IgA transcripts than the noninfected chickens, irrespective of diet composition (Figure 5). Feed supplementation with plant extracts therefore did not interfere with chicken humoral immune response to *S.* Enteritidis.

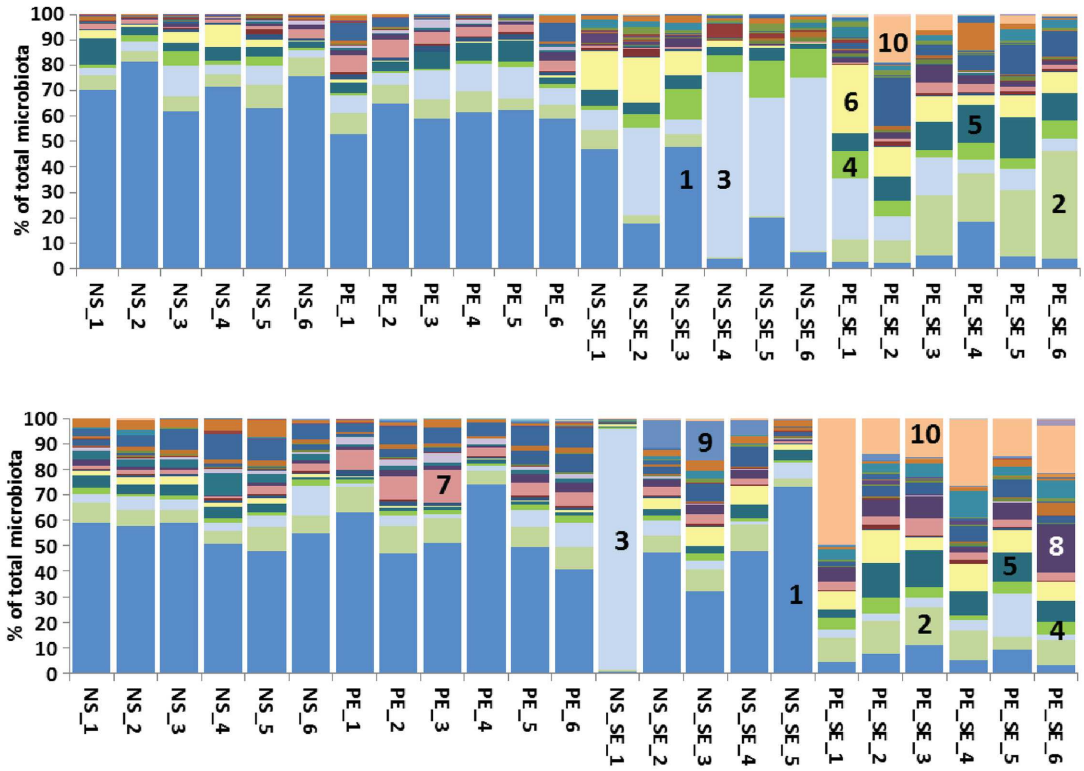

Figure 6. Composition of chicken microbiota at genus level. Each column describes microbiota composition in particular chicken identified by age (11 or 21 d age, upper or lower panel, respectively) and provided a nonsuppplemented diet (NS), diet supplemented with plant extracts (PE), provided a nonsuppplemented diet and infected with *S.* Enteritidis (NS_SE), or provided a plant extract supplemented diet and infected with *S.* Enteritidis (PE_SE). 1, *Clostridium* XlVa; 2, *Lachnospiracea*_incertae_sedis; 3, *Escherichia/Shigella*; 4, *Clostridium* XI; 5, *Blautia*; 6, *Flavonifractor*; 7, *Coprococcus*; 8, *Clostridium* IV; 9, *Eubacterium*; and 10, *Faecalibacterium*.

Changes in Microbiota Following Feed Supplementation with Plant Extracts

Since feed supplementation decreased *S.* Enteritidis counts in the cecum, in the last analysis we tested whether the plant extract supplementation could also be effective against other microbiota members. In total, 596,290 sequences were obtained for all the samples. Coverage per sample ranged from 1,479 to 69,189 sequences.

Sequencing of 16S rRNA amplification products showed that chicken microbiota was dominated by representatives of *Firmicutes* and *Proteobacteria*. Representatives of phylum *Bacteroidetes* only rarely formed more than 1% of microbiota. Supplementation with the plant extracts alone was of minor effect on microbiota composition. Out of the majority genera shown in Figure 6, only *Coprococcus* was positively selected by the supplementation with the plant extracts at both time points. Infection with *S.* Enteritidis affected microbiota composition at 4 d postinfection, i.e., in 11-day-old chickens, since in 4 chickens, *Escherichia coli* formed 30 to 70% of all microbiota. A significantly higher abundance of *Escherichia coli* and *Clostridium* XI occurred at the expense of *Clostridium* XlVa and *Lachnospiracea*_incertae_sedis which were lowered in *S.* Enteritidis infected chickens in comparison to noninfected controls. However, the combined effect of both *S.* Enteritidis infection and plant extract supplementation resulted in the most extensive changes in microbiota composition, both at 4 and 14 d postinfection. Microbiota of *S.* Enteritidis infected chickens fed a diet with plant extracts was characteristic with significantly increased levels of *Lachnospiracea*_incertae_sedis, *Clostridium* XI, *Blautia*, *Flavonifractor*, *Coprococcus*, *Clostridium* IV, *Lactobacillus*, and *Faecalibacterium*, and a decreased abundance of *Clostridium* XlVa) in comparison to microbiota of the noninfected chickens (Figure 6).

DISCUSSION

In this study we were interested in the anti-inflammatory effects of plant extracts from *Curcuma* and *Scutellaria* after heat stress or *S.* Enteritidis infection. Although the heat stress stimulated only a minor inflammatory response in the cecum, the scope of inflammation corresponded with previously reported moderate influx of heterophils into the intestinal tract of heat stressed chickens (Quinteiro-Filho et al., 2012) or induction of IL6 and IL1 in the heat stressed cell line (Prakasam et al., 2013). More importantly, plant extract supplementation lowered this inflammation to levels observed in the cecum of control non-stressed chicken. However, since even the difference between control and heat stressed chickens fed with the

same basal diet was not statistically significant, the protective effect posed by the plant extract supplementation also could not reach statistical significance.

Infections of chickens with nontyphoid serovars of *Salmonella* induce moderate inflammatory response in the cecum of young chickens (Berndt et al., 2007; Matulova et al., 2012b; Matulova et al., 2013). This inflammatory response was prevented by the supplementation of chicken feed with *Curcuma* and *Scutellaria* extracts without any negative effects on BW or humoral immune response. Absence of inflammation also allow for normal gut functions (Varmuzova et al., 2014). The positive effect of plant extract supplementation for chicken gut health was likely a combination of 3 different events: 1) its direct effect on eukaryotic cells as observed in the heat stressed chickens, 2) suppressive effect on *S.* Enteritidis growth, and 3) modification of gut microbiota. The suppressive effect of various plant extracts at various inflammatory markers has been repeatedly reported (Jacob et al., 2007; Sun et al., 2011; Lee et al., 2013; Kamboh et al., 2013). Similarly, direct effect of plant flavonoids against orally administered pathogens such as *Eimeria*, *Clostridium perfringens*, and *Salmonella* Gallinarum was also described (Jung et al., 2010; Kim et al., 2013; Lee et al., 2013), although plant extract supplementation did not protect against intranasal infection with *Escherichia coli* (Peek et al., 2013) indicating the importance of the same localization of plant extract administration and pathogen colonisation.

Changes in the composition of gut microbiota following supplementation of the diet with plant extracts have been reported. However, the majority of these studies used bacterial culture for the description of changes in microbiota composition which therefore limited the information only for the species selected for culture (Viveros et al., 2011; Liu et al., 2014). An understanding of the general relationship between plant extract supplementation and microbiota composition is important for at least two reasons. First, microbiota members may modify and metabolise plant flavonoids which may affect their absorption and biological activity (Manach et al., 1997; Felgines et al., 2000; Iqbal and Zhu, 2009; Rupasinghe et al., 2010). Second, certain microbiota members such as *Faecalibacterium prausnitzii* were reported to decrease gut inflammation (Sokol et al., 2008; Qiu et al., 2013; Zhang et al., 2014) and if these are positively selected by the plant extract, this may provide additional benefit for a host. *Curcuma* and *Scutellaria* extracts used in this study did not affect microbiota composition extensively in the absence of any other experimental factor. However, if provided to the chickens infected with *S.* Enteritidis, the combined effect of plant extract supplementation and *S.* Enteritidis infection resulted in a modification of gut microbiota selecting for a higher abundance of several genera including *Lactobacillus* and *Faecalibacterium*, both bacterial genera considered as those with a positive effect on gut health (Laval et al., 2015). The requirement for a combination of the 2 factors could be explained by a combination of minor inflammation induced by *S.* Enteritidis even in the presence of plant extract supplementation followed by changes in the availability of iron or alterenative electron acceptors for bacterial respiration (Raffatellu et al., 2009; Winter et al., 2010; Thiennimitr et al., 2011). We described a minor increase in *Lactobacillus* abundance in *S.* Enteritidis infected chickens in our recent paper (Videnska et al., 2013). However, we are aware of the fact that the combined effect of plant extract supplementation and *S.* Enteritidis infection at microbiota composition was rather unexpected. An appropriate conclusion should therefore be that *Curcuma* and *Scutellaria* extracts did not extensively affect microbiota composition in chickens up to 3 wk age in the absence of any other stimulus and the recorded combined effect of plant extract supplementation and *S.* Enterititidis infection will have to be verified in additional studies. Despite this we can conclude that supplementation with *Curcuma* and *Scutellaria* plant extracts decreased inflammation in the chicken cecum induced by *S.* Enteritidis infection without any adverse effects on chicken growth or humoral immune response and may be used as feed additivies with beneficial effects on chicken performance.

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Competing interests

The use of *Scutellaria* extract in animal feed is a subject of French patent application FR 14/51501 and PCT application PCT/FR2015/050450.

REFERENCES

Berndt, A., A. Wilhelm, C. Jugert, J. Pieper, K. Sachse, and U. Methner. 2007. Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. Infect. Immun. 75:5993–6007.

Castanon, J. I. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86:2466–2471.

Chen, Y., N. Lu, Y. Ling, Y. Gao, L. Wang, Y. Sun, Q. Qi, F. Feng, W. Liu, W. Liu, Q. You, and Q. Guo. 2009. Wogonoside inhibits lipopolysaccharide-induced angiogenesis in vitro and in vivo via toll-like receptor 4 signal transduction. Toxicology 259:10–17.

Cook, N. C., and S. Samman. 1996. Flavonoids - Chemistry, metabolism, cardioprotective effects, and dietary sources. J. Nutr. Biochem. 7:66–76.

Cox, N. A., J. A. Cason, and L. J. Richardson. 2011. Minimization of *Salmonella* contamination on raw poultry. Annu. Rev. Food Sci. Technol. 2:75–95.

Crhanova, M., H. Hradecka, M. Faldynova, M. Matulova, H. Havlickova, F. Sisak, and I. Rychlik. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella enterica* serovar enteritidis infection. Infect. Immun. 79:2755–2763.

De Boever, S., C. Vangestel, P. De Backer, S. Croubels, and S. U. Sys. 2008. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. Vet. Immunol. Immunopathol. 122:312–317.

Felgines, C., O. Texier, C. Morand, C. Manach, A. Scalbert, F. Regerat, and C. Remesy. 2000. Bioavailability of the flavanone naringenin and its glycosides in rats. Am. J. Physiol Gastrointest. Liver Physiol. 279:G1148–G1154.

Ferrandiz, M. L., and M. J. Alcaraz. 1991. Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. Agents Actions 32:283–288.

Fraser, R. W., N. T. Williams, L. F. Powell, and A. J. Cook. 2010. Reducing *Campylobacter* and *Salmonella* infection: Two studies of the economic cost and attitude to adoption of on-farm biosecurity measures. Zoonoses Public Health 57:e109–e115.

Friesenecker, B., A. G. Tsai, and M. Intaglietta. 1995. Cellular basis of inflammation, edema and the activity of Daflon 500 mg. Int. J. Microcirc. Clin. Exp. 15:17–21.

Goliomytis, M., H. Orfanou, E. Petrou, M. A. Charismiadou, P. E. Simitzis, and S. G. Deligeorgis. 2014. Effect of hesperidin dietary supplementation on hen performance, egg quality and yolk oxidative stability. Br. Poult. Sci. 55:98–104.

Hanasaki, Y., S. Ogawa, and S. Fukui. 1994. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. Free Radic. Biol. Med. 16:845–850.

Heres, L., B. Engel, H. A. Urlings, J. A. Wagenaar, and F. van Knapen. 2004. Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by *Campylobacter* and *Salmonella*. Vet. Microbiol. 99:259–267.

Iqbal, M. F., and W. Y. Zhu. 2009. Bioactivation of flavonoid diglycosides by chicken cecal bacteria. FEMS Microbiol. Lett. 295:30–41.

Jacob, A., R. Wu, M. Zhou, and P. Wang. 2007. Mechanism of the anti-inflammatory effect of curcumin: PPAR-gamma activation. PPAR Res. 2007e:89369.

Jiang, Z. Y., S. Q. Jiang, Y. C. Lin, P. B. Xi, D. Q. Yu, and T. X. Wu. 2007. Effects of soybean isoflavone on growth performance, meat quality, and antioxidation in male broilers. Poult. Sci. 86:1356–1362.

Jung, B. G., J. H. Ko, and B. J. Lee. 2010. Dietary supplementation with a probiotic fermented four-herb combination enhances immune activity in broiler chicks and increases survivability against *Salmonella* Gallinarum in experimentally infected broiler chicks. J. Vet. Med. Sci. 72:1565–1573.

Kamboh, A. A., S. Q. Hang, M. Bakhetgul, and W. Y. Zhu. 2013. Effects of genistein and hesperidin on biomarkers of heat stress in broilers under persistent summer stress. Poult. Sci. 92:2411–2418.

Kamboh, A. A., and W. Y. Zhu. 2014. Individual and combined effects of genistein and hesperidin on immunity and intestinal morphometry in lipopolysacharide-challenged broiler chickens. Poult. Sci. 93:2175–2183.

Kim, D. K., H. S. Lillehoj, S. H. Lee, S. I. Jang, E. P. Lillehoj, and D. Bravo. 2013. Dietary *Curcuma longa* enhances resistance against *Eimeria maxima* and *Eimeria tenella* infections in chickens. Poult. Sci. 92:2635–2643.

Korbecki, J., I. Baranowska-Bosiacka, I. Gutowska, and D. Chlubek. 2013. The effect of reactive oxygen species on the synthesis of prostanoids from arachidonic acid. J. Physiol. Pharmacol. 64:409–421.

Laughton, M. J., P. J. Evans, M. A. Moroney, J. R. Hoult, and B. Halliwell. 1991. Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. Biochem. Pharmacol. 42:1673–1681.

Laval, L., R. Martin, J. Natividad, F. Chain, S. Miquel, C. D. de Maredsous, S. Capronnier, H. Sokol, E. Verdu, H. van, V. L. Bermudez-Humaran, T. Smokvina, and P. Langella. 2015. *Lactobacillus rhamnosus* CNCM I-3690 and the commensal bacterium *Faecalibacterium prausnitzii* A2–165 exhibit similar protective effects to induced barrier hyper-permeability in mice. Gut Microbes 6:1–9.

Lee, S. H., H. S. Lillehoj, S. I. Jang, E. P. Lillehoj, W. Min, and D. M. Bravo. 2013. Dietary supplementation of young broiler chickens with capsicum and turmeric oleoresins increases resistance to necrotic enteritis. Br. J. Nutr. 110:840–847.

Li, Y. P., D. D. Bang, K. J. Handberg, P. H. Jorgensen, and M. F. Zhang. 2005. Evaluation of the suitability of six host genes as internal control in real-time RT-PCR assays in chicken embryo cell cultures infected with infectious bursal disease virus. Vet. Microbiol. 110:155–165.

Liu, H. N., Y. Liu, L. L. Hu, Y. L. Suo, L. Zhang, F. Jin, X. A. Feng, N. Teng, and Y. Li. 2014. Effects of dietary supplementation of quercetin on performance, egg quality, cecal microflora populations, and antioxidant status in laying hens. Poult. Sci. 93:347–353.

Manach, C., C. Morand, C. Demigne, O. Texier, F. Regerat, and C. Remesy. 1997. Bioavailability of rutin and quercetin in rats. FEBS Lett. 409:12–16.

Matulova, M., H. Havlickova, F. Sisak, and I. Rychlik. 2012a. Vaccination of chickens with Salmonella Pathogenicity Island (SPI) 1 and SPI2 defective mutants of *Salmonella enterica* serovar Enteritidis. Vaccine 30:2090–2097.

Matulova, M., J. Rajova, L. Vlasatikova, J. Volf, H. Stepanova, H. Havlickova, F. Sisak, and I. Rychlik. 2012b. Characterization of chicken spleen transcriptome after infection with *Salmonella enterica* serovar Enteritidis. PLoS ONE 7:e48101.

Matulova, M., K. Varmuzova, F. Sisak, H. Havlickova, V. Babak, K. Stejskal, Z. Zdrahal, and I. Rychlik. 2013. Chicken innate immune response to oral infection with *Salmonella enterica* serovar Enteritidis. Vet. Res. 44:e37.

Methner, U., P. A. Barrow, D. Gregorova, and I. Rychlik. 2004. Intestinal colonisation-inhibition and virulence of *Salmonella phoP*, *rpoS* and *ompC* deletion mutants in chickens. Vet. Microbiol. 98:37–43.

Mughini-Gras, L., R. Enserink, I. Friesema, M. Heck, Y. van Duynhoven, and W. van Pelt. 2014. Risk factors for human salmonellosis originating from pigs, cattle, broiler chickens and egg laying hens: A combined case-control and source attribution analysis. PLoS ONE 9:e87933.

Peek, H. W., S. B. Halkes, M. M. Tomassen, J. J. Mes, and W. J. Landman. 2013. In vivo screening of five phytochemicals/extracts and a fungal immunomodulatory protein against colibacillosis in broilers. Avian Pathol. 42:235–247.

Prakasam, R., M. Fujimoto, R. Takii, N. Hayashida, E. Takaki, K. Tan, F. Wu, S. Inouye, and A. Nakai. 2013. Chicken IL-6 is a heat-shock gene. FEBS Lett. 587:3541–3547.

Qiu, X., M. Zhang, X. Yang, N. Hong, and C. Yu. 2013. Faecalibacterium prausnitzii upregulates regulatory T cells and anti-inflammatory cytokines in treating TNBS-induced colitis. J. Crohns Colitis 7:e558–e568.

Quinteiro-Filho, W. M., A. V. Gomes, M. L. Pinheiro, A. Ribeiro, V. Ferraz-de-Paula, C. S. Astolfi-Ferreira, A. J. Ferreira, and J. Palermo-Neto. 2012. Heat stress impairs performance and induces intestinal inflammation in broiler chickens infected with *Salmonella* Enteritidis. Avian Pathol. 41:421–427.

Raffatellu, M., M. D. George, Y. Akiyama, M. J. Hornsby, S. P. Nuccio, T. A. Paixao, B. P. Butler, H. Chu, R. L. Santos, T. Berger, T. W. Mak, R. M. Tsolis, C. L. Bevins, J. V. Solnick, S. Dandekar, and A. J. Baumler. 2009. Lipocalin-2 resistance confers an advantage to *Salmonella enterica* serotype Typhimurium for growth and survival in the inflamed intestine. Cell Host Microbe 5:476–486.

Rupasinghe, H. P., C. M. Ronalds, B. Rathgeber, and R. A. Robinson. 2010. Absorption and tissue distribution of dietary quercetin and quercetin glycosides of apple skin in broiler chickens. J. Sci. Food Agric. 90:1172–1178.

Siler, B., S. Zivkovic, T. Banjanac, J. Cvetkovic, Z. J. Nestorovic, A. Ciric, M. Sokovic, and D. Misic. 2014. Centauries as underestimated food additives: Antioxidant and antimicrobial potential. Food Chem. 147:367–376.

Sokol, H., B. Pigneur, L. Watterlot, O. Lakhdari, L. G. Bermudez-Humaran, J. J. Gratadoux, S. Blugeon, C. Bridonneau, J. P. Furet, G. Corthier, C. Grangette, N. Vasquez, P. Pochart, G. Trugnan, G. Thomas, H. M. Blottiere, J. Dore, P. Marteau, P. Seksik, and P. Langella. 2008. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc. Natl. Acad. Sci. 105:16731–16736.

Sun, Z. J., G. Chen, W. Zhang, X. Hu, Y. Liu, Q. Zhou, L. X. Zhu, and Y. F. Zhao. 2011. Curcumin dually inhibits both mammalian target of rapamycin and nuclear factor-kappaB pathways through a crossed phosphatidylinositol 3-kinase/Akt/IkappaB kinase complex signaling axis in adenoid cystic carcinoma. Mol. Pharmacol. 79:106–118.

Surai, P. F. 2014. Polyphenol compounds in the chicken/animal diet: From the past to the future. J. Anim. Physiol. Anim Nutr. (Berlin) 98:19–31.

Thiennimitr, P., S. E. Winter, M. G. Winter, M. N. Xavier, V. Tolstikov, D. L. Huseby, T. Sterzenbach, R. M. Tsolis, J. R. Roth, and A. J. Baumler. 2011. Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. Proc. Natl. Acad. Sci. 108:17480–17485.

Varmuzova, K., M. E. Matulova, A. Sebkova, Z. Sekelova, H. Havlickova, F. Sisak, V. Babak, and I. Rychlik. 2014. The early innate response of chickens to *Salmonella enterica* is dependent on the presence of O-antigen but not on serovar classification. PLoS ONE 9:e96116.

Videnska, P., F. Sisak, H. Havlickova, M. Faldynova, and I. Rychlik. 2013. Influence of *Salmonella enterica* serovar Enteritidis infection on the composition of chicken cecal microbiota. BMC Vet. Res. 9:140.

Viveros, A., S. Chamorro, M. Pizarro, I. Arija, C. Centeno, and A. Brenes. 2011. Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. Poult. Sci. 90:566–578.

Winter, S. E., P. Thiennimitr, M. G. Winter, B. P. Butler, D. L. Huseby, R. W. Crawford, J. M. Russell, C. L. Bevins, L. G. Adams, R. M. Tsolis, J. R. Roth, and A. J. Baumler. 2010. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. Nature 467:426–429.

Zhang, M., X. Qiu, H. Zhang, X. Yang, N. Hong, Y. Yang, H. Chen, and C. Yu. 2014. *Faecalibacterium prausnitzii* inhibits interleukin-17 to ameliorate colorectal colitis in rats. PLoS ONE 9: e109146.