

Immune Response of Chicken Gut to Natural Colonization by Gut Microflora and to *Salmonella enterica* Serovar Enteritidis Infection[∇]

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In commercial poultry production, there is a lack of natural flora providers since chickens are hatched in the clean environment of a hatchery. Events occurring soon after hatching are therefore of particular importance, and that is why we were interested in the development of the gut microbial community, the immune response to natural microbial colonization, and the response to *Salmonella enterica* serovar Enteritidis infection as a function of chicken age. The complexity of chicken gut microbiota gradually increased from day 1 to day 19 of life and consisted of *Proteobacteria* and *Firmicutes*. For the first 3 days of life, chicken cecum was protected by increased expression of chicken β -defensins (i.e., gallinacins 1, 2, 4, and 6), expression of which dropped from day 4 of life. On the other hand, a transient increase in interleukin-8 (IL-8) and IL-17 expression could be observed in chicken cecum on day 4 of life, indicating physiological inflammation and maturation of the gut immune system. In agreement, the response of chickens infected with *S. Enteritidis* on days 1, 4, and 16 of life shifted from Th1 (characterized mainly by induction of gamma interferon [IFN- γ] and inducible nitric oxide synthase [iNOS]), observed in younger chickens, to Th17, observed in 16-day-old chickens (characterized mainly by IL-17 induction). Active modification of chicken gut microbiota in the future may accelerate or potentiate the maturation of the gut immune system and increase its resistance to infection with different pathogens.

Salmonella enterica is one of the major causes of human food-borne gastroenteritis worldwide, and since poultry are considered to be the most important source of *S. enterica* for humans, measures of how to limit *S. enterica* prevalence in poultry are continuously being sought. The already-in-use measures aiming at the reduction of *S. enterica* prevalence in poultry include strict hygienic standards and vaccination with attenuated or inactivated vaccines. However, although hygienic standards are important for *S. enterica* control, at the same time, this represents an issue. Chickens for commercial production are hatched in a clean environment, and unlike all other farm animals, chickens will never get into contact with adult birds to become colonized by the healthy microflora of adults. Colonization of mucosal surfaces in newly hatched chickens is therefore a matter of coincidence, and if a bacterial pathogen appears in the environment, the sterile intestinal tract of a newly hatched chicken represents an empty ecological niche enabling such a pathogen essentially unrestricted multiplication followed by prolonged colonization. This is the reason why the use of competitive exclusion (CE) products enabling early rapid colonization of chickens with healthy adult gut microbiota has been successfully tested in poultry (18, 20). The positive effect of CE products has been explained by the ability of bacteria present in these products to compete directly with pathogens and also to stimulate maturation of the gut immune system of newly hatched chickens.

The interaction between the immune system of the gut and commensal microbiota in chickens starts immediately after hatching and leads to a low level of inflammation characterized by increased interleukin-8 (IL-8) expression (2). This results in the infiltration of heterophils and lymphocytes into the lamina propria or the gut epithelium and normalization of the gut immune system (3, 16, 30). Infiltrating lymphocytes develop further, depending on the gut flora composition, either in terms of a decreasing ratio of $\alpha\beta$ to $\gamma\delta$ T lymphocytes in the lamina propria or the gut epithelium (15) or in terms of changes in $\alpha\beta$ T-cell receptor repertoires (21). In mice, but not in chickens so far, gut microflora has been reported to induce the Th1 and Th17 arms of the immune response, with IL-17 playing an important role in the maturation of the murine gut immune system (9, 12). Interestingly, IL-17 has been shown to be important for defense against bacterial and fungal pathogens (5), and in mice, IL-17 is induced also after *S. enterica* serovar Typhimurium infection (25). This means that the patterns of immune response to commensals and pathogens may overlap, the former being a subset of the latter. A detailed understanding of these responses may then allow active modification of gut microbiota composition, especially in the early days of life, and potentiation of the immune response against particular bacterial or fungal pathogens.

In this study, we were therefore interested in the development of chicken cecum microflora and the corresponding maturation of the gut immune system. We found out that chickens responded to the natural colonization of cecum by an increased expression of IL-8 and IL-17 in the first week of life. In the second part of this study, we infected chickens with *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) before,

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during, and after the IL-8 and IL-17 induction and found out that chickens infected before and during microbiota-induced IL-8 and IL-17 responded to *S. Enteritidis* infection more through Th1, while birds infected after this time point, on day 16 of life, responded more through the Th17 branch of the immune response. We therefore concluded that the transient induction of proinflammatory cytokines by gut microbiota resulted in an activation and normalization of the innate immune system in the chicken gut and also a modified immune response and increased resistance to *S. Enteritidis* infection.

MATERIALS AND METHODS

Experimental animals and infection. In all experiments, male ISA Brown newly hatched chickens were used. The chickens were reared in wire cages and allowed free access to water and pathogen-free feed. In the first experiment, three chickens each were sacrificed on days 1, 4, 7, 10, 13, 16, and 19 of life, and the development of natural gut microflora cytokine and gene expression in cecum was monitored. In the second experiment, three chickens each were sacrificed on days 1, 2, 3, 4, 5, and 7 of life, and cytokine gene expression in cecum was monitored. In the last experiment, 1-, 4-, and 16-day-old birds (22 birds in each group) were infected orally with 1×10^6 CFU of the wild-type *S. Enteritidis* strain 147 spontaneously resistant to nalidixic acid (19) and sacrificed 3 days (6 birds), 10 days (6 birds), and 42 days (10 birds) postinfection, respectively. In addition, in this experiment, three noninfected chickens used as negative controls were sacrificed on days 4, 7, 10, 14, 15, 16, and 19, and six noninfected chickens used as negative controls were sacrificed on days 26, 47, and 58.

Cecal contents were stored at -20°C prior to DNA purification, and cecal wall samples were placed in RNALater solution (Qiagen) and stored at -70°C prior to RNA purification. *S. Enteritidis* counts in the cecum, liver, and spleen were determined after tissue homogenization in peptone water and plating of 10-fold serial dilutions on xylose-lysine-deoxycholate (XLD) agar supplemented with nalidixic acid. Samples negative after direct plating were subjected to enrichment in modified semisolid Rappaport Vassiliadis medium for qualitative *S. Enteritidis* determination. Counts of *S. Enteritidis* samples positive after direct plating were logarithmically transformed. Samples positive only after preenrichment were assigned a value of 1, and negative samples were assigned a value of 0.

16S rRNA gene T-RFLP. For 16S rRNA gene terminal restriction fragment length polymorphism (T-RFLP) analysis, cecal contents were homogenized by using zirconia silica beads (BioSpec Products) in a MagNALyzer (Roche), and DNA was extracted by a QIAamp DNA stool minikit according to the manufacturer's instructions (Qiagen). The purified DNA was used as a template in PCR amplification with fluorescently labeled universal primers for 16S rRNA genes 27F (6-carboxyfluorescein [FAM]-5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' GGY TAC CTT GTT ACG ACT T 3'). Following PCR, the amplification products were digested with HaeIII restriction endonuclease, and the resulting fragments were separated and identified by capillary electrophoresis using an ABI310 genetic analyzer (Applied Biosystems).

Quantitative real-time PCR. Cecal wall samples stored in RNALater were homogenized in exactly the same way as the cecal content samples, and total RNA was purified using TRI reagent (Molecular Research Center). The purity and concentration of RNA were determined spectrophotometrically, and 1 μg of RNA was immediately reverse transcribed by using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) and oligo(dT) primers. The resulting cDNA was $10\times$ diluted in sterile distilled water and used as a template in real-time PCR or stored at -20°C until used. Real-time PCR was performed in microplates with a LightCycler II (Roche) and QuantiTect SYBR green PCR kit (Qiagen). The mRNA levels of 18 cytokines, chemokines, or immune-associated proteins (IL-2, -4, -5, -6, -10, -12p35, and -12p40; granulocyte-macrophage colony-stimulating factor [GM-CSF]; NRAMP [natural resistance macrophage-associated protein]; IL-15, -1 β , -8, -17, -18, and -22; tumor necrosis factor alpha [TNF- α ; also called LITAF]; gamma interferon [IFN- γ]; inducible nitric oxide synthase [iNOS]; and gallinacins 1, 2, 4, and 6) were determined. In addition, expression of three housekeeping genes, coding for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), TATA-binding protein (TBP), and ubiquitin (UB), was determined and used for data normalization (6, 13). Primers for cytokine genes were either described previously (4, 26) or newly designed with Primer3 software (<http://frodo.wi.mit.edu/primer3/>), and all primer sequences are listed in Table 1. Each sample was subjected to reverse transcription-PCR (RT-PCR) in duplicate, and the mean values were used for subsequent analysis. The threshold cycle (C_T) values of genes of interest were normalized to an average C_T value

TABLE 1. List of primers used for the chicken cytokine mRNA quantification

Primer	Sequence (5'→3')	Source or reference
IL- β For	GAAGTGCTTCGTGCTGGAGT	This study
IL- β Rev	ACTGGCATGCCCCAGTTC	
IL-8For	ATGAACGCAAGCTTGGAGCT	26
IL-8Rev	GCAGCTCATTCCCCATCTT	
IL-12 β For	TGGTCCACGCTTTGCAGAT	4
IL-12 β Rev	AAGGTTAAGGCGTGGCTTCTTA	
IL-17For	TATCAGCAAACGCTCACTGG	This study
IL-17Rev	AGTTCACGCACCTGGAATG	
IL-18For	ACGTGGCAGCTTTTGAAGAT	26
IL-18Rev	CGGTTGGTTTTGTAACAGTG	
IL-22For	CAGACTCATCGTGCAGAAA	This study
IL-22Rev	GGTACCTCTCCTTGGCCCTCT	
IFN- γ For	GCCGCACATCAAACACATATCT	4
IFN- γ Rev	TGAGACTGGCTCCTTTTCCTT	
TNF- α For	AATTTGCAGGTGTTTCTCG	26
TNF- α Rev	TATGAAGGTGGTGCAGATGG	
iNOSFor	GAACAGCCAGCTCATCCGATA	4
iNOSRev	CCCAAGCTCAATGCACAACTT	
Gal1For	GAAATGCTCAAGATTTTACTCTG	This study
Gal1Rev	GAAGTTTCCAGAAGCGAGAAG	
Gal2For	AGGATTCTTTACCTGCTTTTCTCT	This study
Gal2Rev	AGCTTCCGACTTTGATTAGATG	
Gal4For	GTGTCTGTAGGTGCAGCAACTCT	This study
Gal4Rev	ATTGCATGTGATATCTTGGAGAAC	
Gal6For	TACTGGAGAAGGGAGACAGAAG	This study
Gal6Rev	TGATATCTACAAGCATGAATAGGG	
GAPDHFor	CCTGCATCTGCCACTTT	6
GAPDHRev	GGCACGCCATCACTATC	
TBPFor	TAGCCCGATGATGCCGTAT	13
TBPRev	GTTCCCTGTGTCGCTTGC	
UBFor	GGATGCAGATCTTCGTGAAA	6
UBRev	CTTGCCAGCAAAGATCAACCTT	

of the housekeeping genes, and the relative expression of each representative was calculated as $2^{-\Delta C_T}$. Cytokine expression in the cecum was determined in three independent animal infections, and in the figures, we present results combined from all of these experiments.

ELISA detection of anti-Salmonella LPS antibodies. To detect *Salmonella* anti-lipopolysaccharide (anti-LPS) antibodies, the commercial Flockscreen *Salmonella enterica* serovar Enteritidis antibody enzyme-linked immunosorbent assay (ELISA) kit (x-OvO, United Kingdom) was used to detect serum antibodies in birds infected with *S. Enteritidis* on day 1, 4, or 16 of life. Forty-two days postinfection, serum was obtained from the birds, and the ELISA was performed exactly as recommended by the manufacturer, except we diluted the serum only 1:5 in the dilution solution provided by the kit.

Statistics. One-way analysis of variance (ANOVA) was used to analyze cytokine expression levels at different chicken ages with Tukey's *post hoc* test. Student's *t* test was used for the analysis of cytokine expression levels in *Salmonella*-infected and noninfected chickens on any particular day. All statistical analyses were performed using SPSS v. 14.0 software. For clarity, in Fig. 2, 4, and 5, we do not show standard deviation; instead, we indicate statistically significant differences.

RESULTS

Chicken gut flora development. Using a 16S rRNA gene T-RFLP, we determined the development of gut microbiota in chickens, from the day of hatching until day 19 of life (Fig. 1A). The complexity of gut microbiota gradually increased, with the most dramatic development occurring during the first week of life. The lowest complexity of cecal microbiota was observed on day 1, when the flora usually consisted of five different species. Three days later, the number of cecal microbiota in-

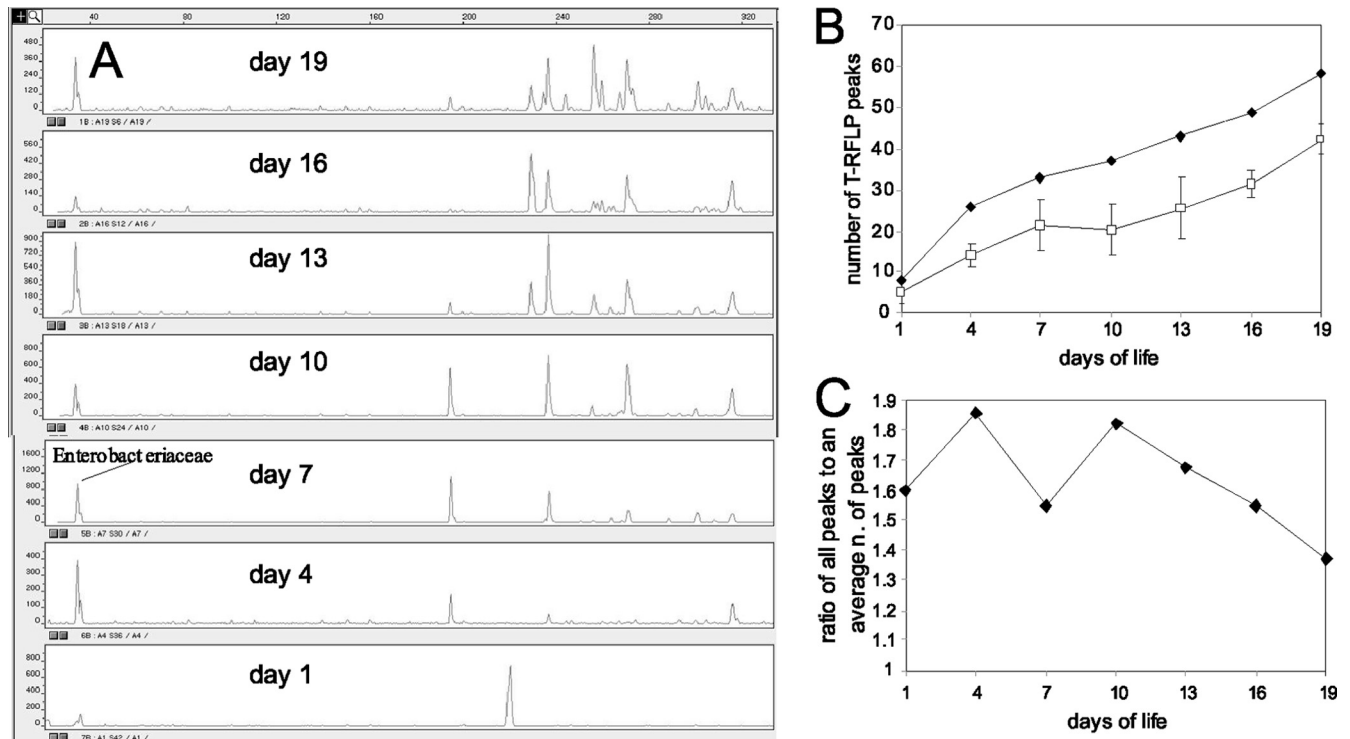


FIG. 1. Cecal microflora development in chickens. (A) T-RFLP analysis; (B) number of T-RFLP peaks. Solid symbols, total number of unique peaks identified in three different birds at each time point; open symbols, average number of peaks ± standard deviation (SD) per individual bird. (C) Ratio of the total number of peaks to an average number of peaks.

creased to 14, which gradually increased to approximately 42 peaks characterizing microbiota in 19-day-old chickens (Fig. 1B). When the numbers of all of the different peaks observed in the three birds tested at each sampling time were counted and the ratios of all different peaks to an average number of peaks were calculated, the highest ratio, and therefore largest number of differences in gut flora composition of individual birds, was observed during the first 10 days of life. On the other hand, the ratio decreased in the second week of life, indicating that the gut microbiota composition in 19-day-old chickens was closely related (Fig. 1B and C).

Cytokine response to cecum colonization by normal gut flora. Simultaneously with microflora development, we determined the cytokine immune response in chicken cecum. Initially we determined the gene expression of 20 different cytokines and immune-relevant genes. Out of these, expression of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, and GM-CSF was quite low in the cecum, with C_T values of >30, and the genes coding for these cytokines were therefore excluded from further detailed analysis. Expression of IL-15 and NRAMP could be detected easily in cecal samples; however, their expression levels did not change significantly, neither in response to natural gut flora colonization nor in response to *S. Enteritidis* infection. Expression of IL-1β, IL-8, IL-17, IL-18, IL-22, TNF-α, IFN-γ, and iNOS changed either in response to normal gut colonization or in response to *S. Enteritidis* infection.

Specific expression profiles in response to gut flora development were observed for IL-8 and IL-17 and partially also for IFN-γ. Expression of IL-8 and IL-17 was characterized by peak

expression on day 4 of life followed by a decrease in gene expression, and from day 10, the expression of these two cytokines did not change any more. The expression profile of IFN-γ in the chicken cecum was different from that of the previous two cytokines since it had been increasing gradually, from the day of hatching until the last measurement on day 58 (Fig. 2).

Expression of chicken β-defensins in cecum in a response to colonization by normal gut flora. Since IL-17 is the cytokine characteristic for the Th17 arm of the immune response, which controls immune response on mucosal surfaces, in the next experiment, we were interested in whether chicken β-defensins (i.e., gallinacins 1, 2, 4, and 6) will follow the same expression profiles as IL-17. All of the gallinacins exhibited very similar expression profiles, which, however, did not overlap with that of IL-17. The gallinacins were highly expressed on the first 3 days of the chicken's life, but then on day 4, their expression dropped considerably (Fig. 3).

Salmonella infection and counts in cecum. Next we were interested in the influence of gut microflora on chicken resistance to *S. Enteritidis* infection. Following previous results, we infected chickens 1, 4, and 16 days old (i.e., before, during, and after the physiological inflammatory response to normal gut flora) and monitored *S. Enteritidis* counts and cytokine responses 3, 10, and 42 days postinfection. When counts of *S. Enteritidis* were determined in the cecum, liver, and spleen of infected chickens, the counts in the cecum did not differ among birds infected at different ages, thus showing that gut microbiota did not influence *S. Enteritidis* cecum colonization. On the

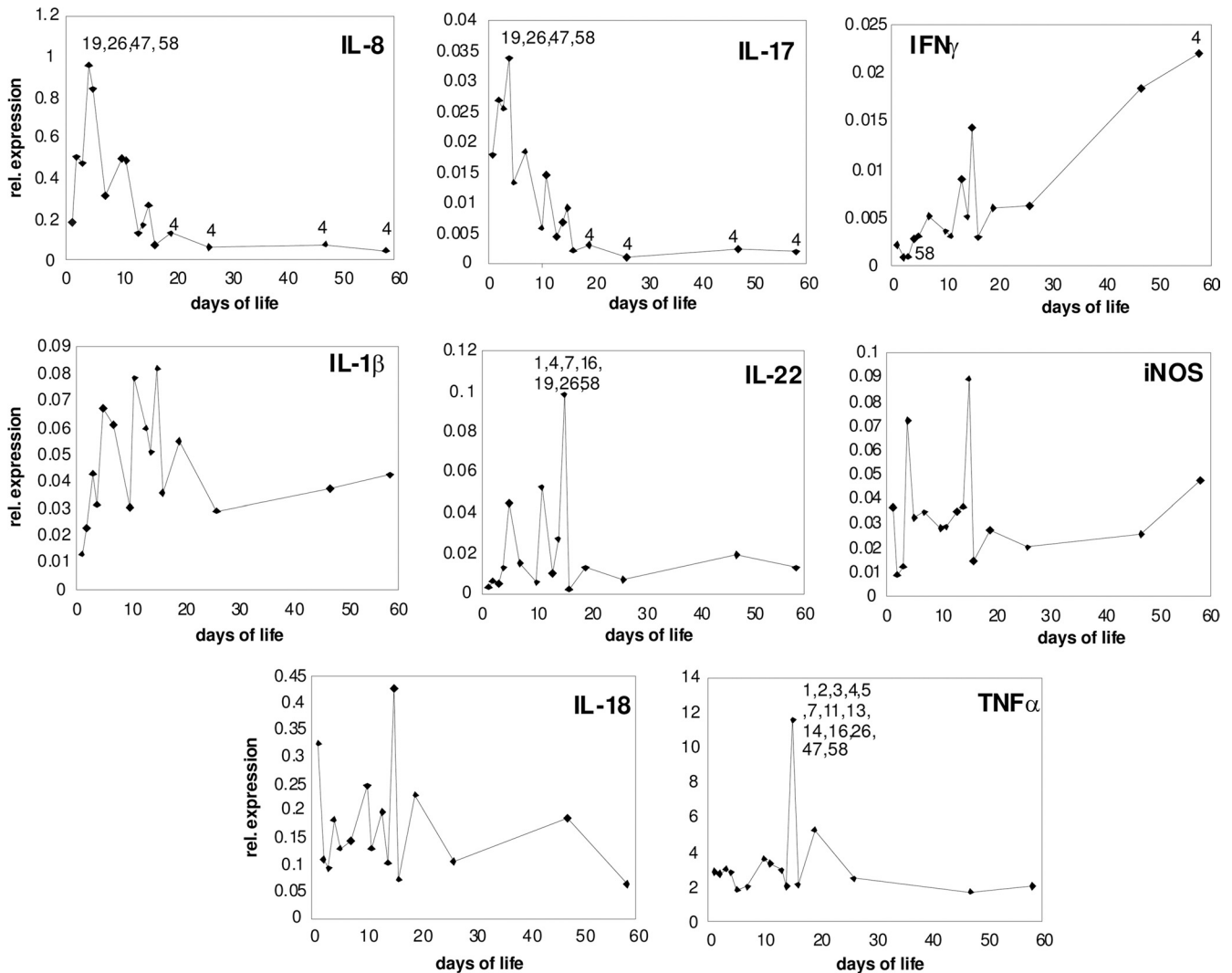


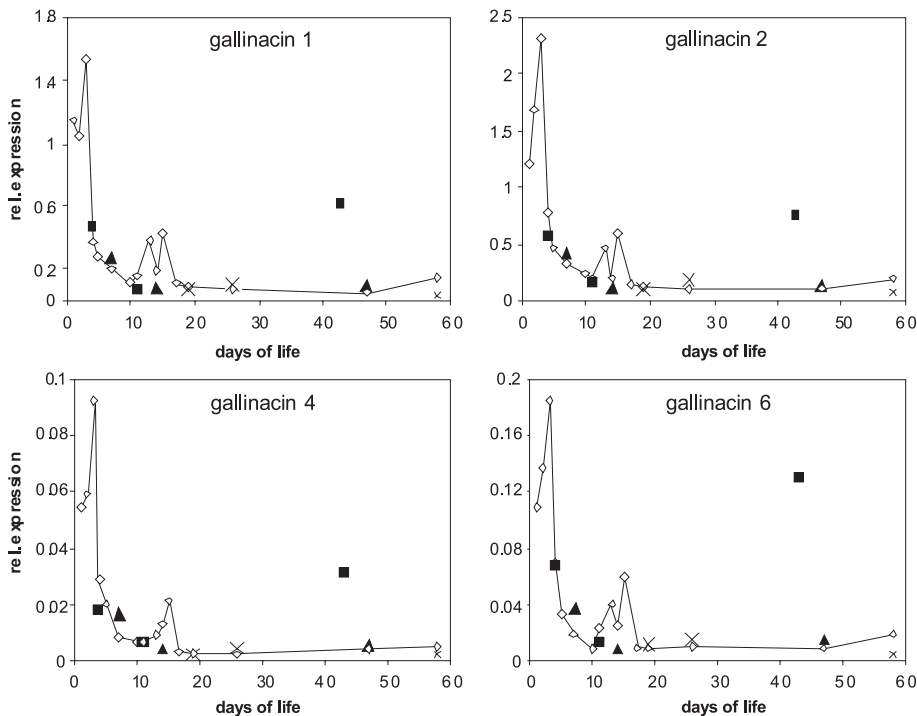
FIG. 2. Cytokine gene expression in the cecum of noninfected chickens. For days 1, 19, 26, 47, and 58, each dot is a mean value of relative (rel.) expression from six chicks. For days 2, 3, 5, 10, 11, 13, 14, 15, and 16, each dot is a mean value from three chicks. For days 4 and 7, each dot represents a mean value from nine chicks. Numbers in the individual panels indicate significant differences in cytokine expression between particular days of life by one-way ANOVA at $P < 0.05$.

other hand, *S. Enteritidis* counts in the liver and spleen showed a negative correlation with the age of infected chickens as the highest *S. Enteritidis* counts were observed in chickens infected at 1 day old, and the lowest *S. Enteritidis* counts in the liver and spleen were observed in chickens infected on day 16 of life (Fig. 4).

Cytokine response of 1-day-old chickens. The infection of 1-day-old chickens led to significantly increased expression of IL-1 β , IL-8, IL-17, IL-18, IL-22, IFN- γ , and iNOS in the cecum compared with that of the noninfected controls at either 3 or 10 days postinfection. Except for IFN- γ and TNF- α , all of the remaining cytokines were maximally expressed 3 days postinfection, and their expression gradually decreased between 10 and 42 days postinfection. Three days postinfection, the highest induction was observed for IL-22 (approximately 60-fold induction), followed by IL-1 β , IFN- γ , and iNOS (each induced around 5-fold) compared with that of the noninfected chickens. Ten days postinfection,

IFN- γ and iNOS were expressed at levels 35 to 40 times higher in *Salmonella*-infected chickens than those in noninfected chickens, and expression of IL-22 and IL-1 β was upregulated ~10-fold. Forty-two days postinfection, IL-17 and IL-1 β still remained expressed approximately 7 times more than in noninfected control chickens, although due to increased variation, this upregulation was no longer statistically significant (Fig. 5).

Cytokine response of 4-day-old-chickens. The infection of 4-day-old chickens led to increased expression of IL-1 β , IL-8, IL-17, IL-18, IL-22, TNF- α , IFN- γ , and iNOS in the cecum compared with the level in the noninfected controls both 3 and 10 days postinfection; however, due to a high variation in gene expression in both infected and noninfected chickens, none of these upregulations has come out as significant. Despite this, IL-1 β , IL-8, IL-17, and IL-22 were maximally expressed 3 days postinfection, and their expression gradually decreased between 10 and 42 days



	day 1	day 2	day 3	day 4	day 5	day 7	day 10	day 11	day 13	day 14	day 15	day 16	day 19	day 26	day 47	day 58
	ANOVA significantly different at P<0.05 from the expression on day															
gal 1	7,10,11,16, 19,26,47,58	7,10,11,16,19, 26,47,58	4,5,7,10,11,13,14, 15,16,19,26,47,58	3	3	1,2,3	1,3	1,2,3	3	3	3	1,3	1,2,3	1,2,3	1,2,3	1,2,3
gal 2		7,10,11,16,19, 26,47	4,5,7,10,11,13,14, 15,16,19,26,47,58	3	3	2,3	2,3	2,3	3	3	3	2,3	2,3	2,3	2,3	2,3
gal 4	7,11,19,26, 47,58	7,10,11,16,19, 26,47,58	4,5,7,10,11,13,14, 15,16,19,26,47,58	3	3	1,2,3	2,3	1,2,3	3	3	3	3	1,2,3	1,2,3	1,2,3	1,2,3
gal 6		7,11,19,26,47 16,19,26,47,58	4,5,7,10,11,13,14, 15,16,19,26,47,58	3	3	3	3	2,3	3	3		3	2,3	2,3	2,3	2,3

FIG. 3. Chicken β -defensin gene expression in noninfected chickens (open diamonds) and chickens infected with *S. Enteritidis* at 1 day old (solid squares), 4 days old (solid triangles), and 16 days old (crosses) and sacrificed 3, 10, and 42 days postinfection. rel., relative. The table under the figure indicates significantly different levels of expression of individual gallinacins (gal 1, 2, 4, or 6) versus those of noninfected chickens at different days of life.

postinfection. IL-18, IFN- γ , iNOS, and TNF- α reached their maximal expression 10 days postinfection. Three days postinfection, the highest induction, compared with noninfected chickens, was observed for IL-22 and iNOS (around

25- and 15-fold, respectively). Ten days postinfection, iNOS and IFN- γ were expressed 15 times higher in *S. Enteritidis*-infected chickens than in noninfected chickens. Forty-two days postinfection, none of the cytokines in *S. Enteritidis*-

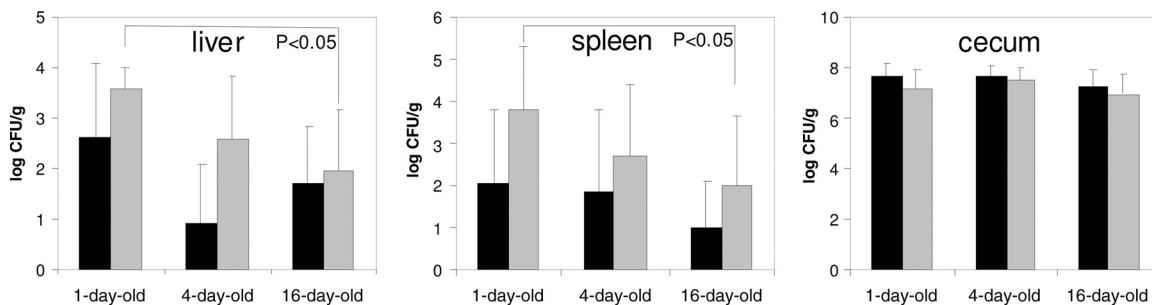


FIG. 4. *S. Enteritidis* counts in liver, spleen, and cecum. Black columns represent average *S. Enteritidis* counts in organs from chickens 3 days postinfection. Gray columns represent average *S. Enteritidis* counts in organs from chickens 10 days postinfection. *S. Enteritidis* counts from spleen, liver, and cecum of infected chickens 42 days postinfection were all negative and therefore are not shown.

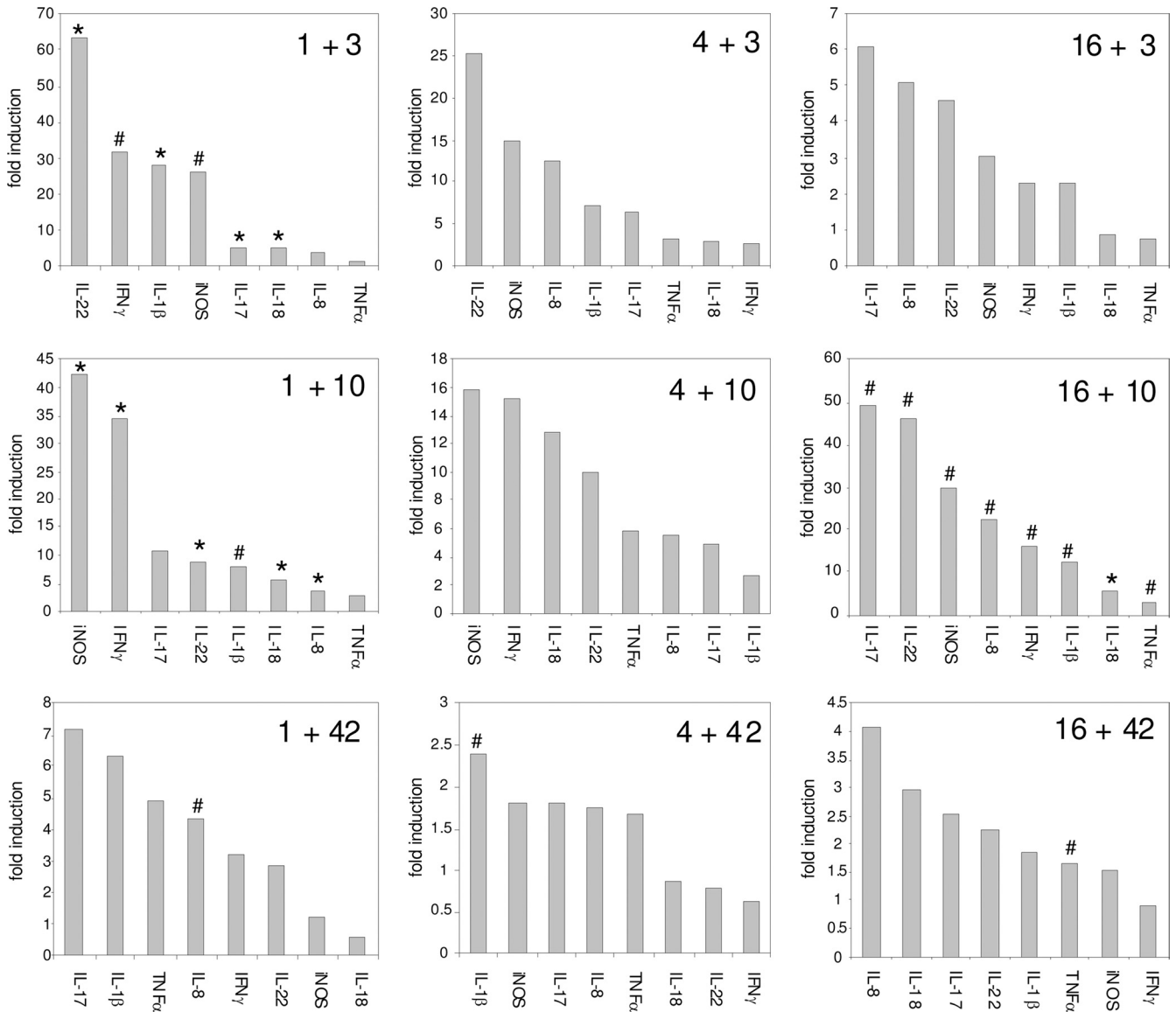


FIG. 5. Relative increase in fold inductions of selected cytokines after infection with *S. Enteritidis*. The following format was used: “1 + 3” represents infection of 1-day-old chickens and determination of cytokine gene expression 3 days postinfection. *, value by *t* test significantly different from that for noninfected controls at $P < 0.01$; #, value by *t* test significantly different at $P < 0.05$.

infected chickens remained expressed more than 2.5 times higher than the levels in noninfected controls (Fig. 5).

Cytokine response of 16-day-old chickens. The infection of 16-day-old chickens led to significantly increased expression of IL-1 β , IL-8, IL-17, IL-22, IFN- γ , iNOS, IL-18, and TNF- α in the cecum compared with that of the noninfected controls, which was restricted only to a sampling time of 10 days postinfection. The highest induction was observed for IL-17 and IL-22 (nearly 50-fold), followed by iNOS (30-fold), IL-8 (25-fold), IFN- γ (15-fold), and IL-1 β (10-fold) (Fig. 5).

Salmonella infection and expression of β -defensins. Independent of the age of the infected chicken or type of cytokine response to the infection, chickens never responded to *S. Enteritidis* by modified expression of gallinacins 1, 2, 4, and 6. The high expression of all of the gallinacins in 1-day-

old chickens 42 days postinfection is unlikely to be associated with *S. Enteritidis* infection since the chickens were virtually free of *S. Enteritidis* at that time, and they did not respond to *S. Enteritidis* infection by increased gallinacin expression 4 and 10 days postinfection (Fig. 3).

Salmonella LPS ELISA. Independent of the age of infected chickens, their infection with *S. Enteritidis* resulted in a significant increase in the titer of anti-LPS antibodies determined in blood serum 42 days postinfection. High differences in responses of individual birds could be observed as characterized by quite a high standard deviation. The high variation observed in individual bird responses was also a reason why the numerically lower titer of antibodies observed in birds infected on day 4 of life came out as insignificant compared with the titer in those infected at day 1 or day 16 of life (Fig. 6).

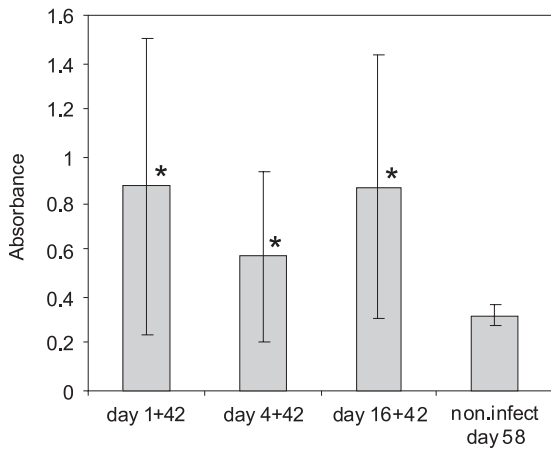


FIG. 6. *Salmonella* LPS ELISA 42 days postinfection of 1-, 4-, and 16-day-old chickens, with 58-day-old noninfected (non.infect) chickens used as a control. The following format was used: “1 + 42” represents infection of 1-day-old chickens and detection of serum antibodies 42 days later. *, value by *t* test significantly different from noninfected controls at *P* < 0.05.

DISCUSSION

In this study, we have shown that complexity of chicken microbiota gradually developed from day 1 to day 19, and that, among younger birds, there was greater individual variation in microbiota composition than among older birds from the same flock. These observations are similar to the other reports on gut flora development in broiler chickens or pigs (17, 29). These observations also indicate that the birds were initially randomly colonized by bacterial species present in the hatchery and sharing the same environment, which then resulted in a convergence of gut microbiota of individual members belonging to the same flock. Our preliminary characterization of individual components of gut microbiota based on the cloning and sequencing of 16S rRNA amplification products (unpublished data) showed that the peak of 34 nucleotides in size was associated with the presence of *Enterobacteriaceae* (phylum *Proteobacteria*), while all the other peaks were associated with the presence of different representatives of the phylum *Firmicutes*, in agreement with previous reports (1, 17, 24, 33).

Chickens that are hatched as immunologically immature only weakly respond to vaccination with an inactivated antigen (11), and their splenic T cells do not proliferate if treated with various mitogens (16). In both of these studies, the maturation of the immune system was observed to start between days 2 and 4 of life. In agreement with these studies, we observed that for the first 3 days of life, chicken cecum was protected from incoming antigens by increased expression of gallinacins 1, 2, 4, and 6. On day 4, peak expression of IL-8 and IL-17 has been observed that indicated minor, transient, inflammation followed by normalization of the gut immune system and suppression of gallinacin expression. Upregulation of IL-8 in response to the colonization by gut microbiota has been reported also by Bar-Shira and Friedman (2). Although we did not investigate this in our study, it is likely that the same physiological inflammation happened also in studies which determined

cellular infiltrates in chicken intestinal tract soon after hatching and in which minor granulocyte and T-lymphocyte infiltrates to cecal lamina propria in chickens on day 4 of life were observed (3, 15, 30). The observed transient inflammation, despite the continuous increase in complexity of gut microbiota, could be caused either by the appearance of gut microbiota with anti-inflammatory activity (9, 28) or, more likely in our opinion, by an adaptation of the gut immune system to contact with microbiota. We also considered whether the transient inflammation could be influenced by nonbacterial antigens present in feed. However, we concluded that this hypothesis is also less likely because in germ-free mice, which uptake feed normally, the inflammation signs and influx of granulocytes and lymphocytes appear only after experimental bacterial colonization (9).

Compared with the response to natural gut colonization, infection by *S. Enteritidis* induced IL-8 and IL-17, however, to a significantly higher level than that induced by normal gut microbiota. Chickens infected by *S. Enteritidis* responded also by increased expression of IL-1 β , IL-22, iNOS, IFN- γ , and IL-18. Except for IL-17 and IL-22, increased expression of the remaining cytokines in chickens in a response to *S. enterica* infection has been reported earlier (4, 31). Although not in chickens, IL-17 and IL-22 have been described as highly inducible by *Salmonella* infection in mice or a human cell line (10, 25, 27). Rather unexpectedly, none of the four gallinacins tested increased in expression in a response to *S. Enteritidis* infection, further indicating that these gallinacins are not part of IL-17 and the Th17 arm of immune response in chickens.

IL-17 and IL-22 are frequently expressed simultaneously (7, 14), although under specific conditions, IL-17 and IL-22 might be expressed independently by different effector T cells (8, 23). Such different conditions were observed also in our study when IL-22 was induced only by *S. Enteritidis* infection, while IL-17 was induced both by *S. Enteritidis* and common gut microbiota. Furthermore, IL-22 was highly induced when the chicks were infected on days 1, 4, and 16 of life, while maximal IL-17 induction in a response to *S. Enteritidis* infection was observed in 16-day-old chickens. IL-17 may regulate immune response since IL-17 receptors have been found on dendritic cells, macrophages, and T lymphocytes (23). On the other hand, the IL-22 receptor has been observed only on nonimmune cells, stimulating them for production of antimicrobial peptides and also stimulating their growth and regeneration during infection (7, 23). Normal microbiota therefore stimulate the proinflammatory response, which due to the absence of IL-22 upregulation, does not result in tissue damage. On the other hand, infection with *S. Enteritidis* resulted in significant inflammation, which also required a strengthening of epithelial cell resistance and healing controlled by IL-22 and independent of primarily the Th1 response observed in 1- and 4-day-old chicks or rather the Th17 response observed in 16-day-old chickens.

We did not observe any direct effect of gut microbiota on *S. Enteritidis* cecum colonization because the same *S. Enteritidis* counts were observed in chickens infected on days 1, 4, and 16 of life. However, gut microbiota clearly had an effect on immune system maturation, which resulted in increased resis-

tance of older birds to *S. Enteritidis* infection. IL-17 induced during the first week of the chicken's life likely resulted in an influx and differentiation of Th17 cells in lamina propria as described in the response to microbiota colonization in mice (12, 22). In response to *S. Enteritidis* infection, mature Th17 cells then produced IL-17 and IL-22 and increased the resistance of epithelial cells by increasing the production of antimicrobial peptides (14) and by recovery of damaged tissue stimulated by IL-22 (32). The increased Th17-dependent resistance in 16-day-old chickens then protected them from extensive *S. Enteritidis* dissemination to the liver and spleen. In addition, the matured Th17 arm of the gut immune system also caused (i) a delayed maximal cytokine response to *S. Enteritidis* from day 3 postinfection in 1-day-old chicks to day 10 postinfection in 16-day-old chickens and (ii) reduced absolute levels of cytokine expression in birds infected on day 16 of life compared with the chickens infected immediately after hatching, as well as (iii) shifted the IFN- γ and iNOS responses of 1- and 4-day-old chickens into the IL-22 and IL-17 responses of 16-day-old chickens.

In this study, we have shown that although gut flora gradually developed in young chickens, the most important events occurred during the first week of life when transient gut inflammation could be detected. For the first 3 days of life, chicken cecum was protected by a high level of expression of chicken β -defensins, and this type of cecum protection was replaced from day 4 of life by normalization of the gut immune system induced by transient expression of proinflammatory cytokines IL-8 and IL-17. This physiological inflammation delineates newly hatched chickens responding primarily by the Th1 type of immune response from birds older than 10 days, which respond to *S. Enteritidis* by the Th17 arm of the immune response. Although microbiota development must have been a rather specific and uncontrolled process in our experiments, it is likely that the same maturation happens in general, as many studies reported either increased cytokine signaling or cellular infiltrates in early days posthatching (2, 3, 15, 30). Such a maturation of the chicken gut immune system leads to increased resistance to *S. Enteritidis* but likely also to other bacterial or protozoan pathogens. It would be interesting to perform similar experiments with controlled colonization of newly hatched chickens, either with complex gut flora preparations originating from healthy birds modeling the natural conditions or with monocultures with known probiotic potential such *Lactobacillus* or *Bifidobacterium*.

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