

Response of the specific immune system on cecal colonization of slow-growing broiler chickens reared on litter contaminated by *Campylobacter jejuni*

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Abbreviated title: Chicken immune system against *Campylobacter*

Summary

The aim of this experiment was to study the relation between the specific immune response and infection of slow-growing broiler chickens reared on straw litter contaminated by *Campylobacter jejuni*. The 11-weeks experiment was carried out in a 45 m² broiler house with 500 chickens from 1 day of age, which had access to an open-air range. Litter contamination was performed before the setting up of the chicks by rearing campylobacter-positive cocks. These birds were also used to contaminate the open-air range during one week before the exit of the chickens, at 5 weeks of age. Blood samples were taken weekly on 20 chickens for anti-campylobacter IgG quantitative measurements on serum. Chickens were then euthanized and cecal contents and bile were collected for *Campylobacter* enumeration and anti-campylobacter IgA quantification, respectively. A high serum anti-campylobacter IgG titer of 110.13 was measured in one-day-old chicks. The IgG antibodies level significantly decreased to 5.60 at 28 days of age which coincided with the onset of *Campylobacter* infection in the flock. A significant reduction of the cecal colonization from 11 weeks of age was correlated with the increase in biliary IgA titers. These results suggest that the 2-3 week's delay

generally observed in poultry production before flock infection by *Campylobacter* may be, at least partly, explained by the protective effect of maternal antibodies. The increase of secretory immune response as bird age may help to limit *Campylobacter* population in the chicken gut.

Keywords: antibody response, *Campylobacter jejuni*, colonization, slow-growing chickens

Introduction

Campylobacter spp. is a major cause of human bacterial enteritis, mainly by consumption of contaminated poultry products. Broiler chicken flocks are frequently infected by this pathogen but they generally do not show any clinical signs. *Campylobacter* is a commensal microorganism in poultry (Hendrixson and DiRita., 2004), colonizing mainly caeca but also cloaca and large intestine. Commensal association between host and bacteria is characterized by the absence of any inflammatory response and the inability of the secretory and systemic immune response to eliminate the bacteria (Van Deun *et al.*, 2008). Nevertheless, *Campylobacter* colonization in chickens induces increased specific IgG, IgA and IgM circulating antibodies (Cawthraw *et al.*, 1994). The efficacy of such antibodies in preventing or limiting infection remains unknown.

Poultry flock infection is generally higher (up to 100%) in organic and free-range production system compared to intensively reared flocks (Newell and Fearnley, 2003). This presumably reflects the level of environmental exposure of such birds as well as the increased age of the birds at slaughter. In Europe, most poultry flocks become infected only 2 to 3 weeks after the placement of chicks into the broiler house (Newell and Fearnley, 2003). It is not well understood if this delay is due to the time needed for *Campylobacter* to come into the broiler house via the environment or to the contamination chick resistance. Sahin *et al.* (2003) actually suggested that anti-campylobacter maternal IgG antibodies may contribute to the lack of *Campylobacter* infection in young broiler chickens, following

experimental infection of 3-day-old chicks with *C. jejuni*. Nevertheless, relation between maternal antibodies and natural flock infection has not been studied.

The aim of this experiment was to study the relation between the specific immune response and *Campylobacter* contamination of slow-growing broiler chickens from free-range production system reared on straw litter naturally contaminated by *Campylobacter jejuni*.

Materials and Methods

The experimental protocols complied with the guidelines of the Animal Care and Use Committee (protocol FUSAGx08/04) of Gembloux Agricultural University.

The experiment was carried out in a 45 m² broiler house with 500 slow-growing JA657 broiler chickens from 1 to 11 weeks of age. Detection of *Campylobacter* according to the ISO 10272 standard was performed on the paper liners from the delivery trays before the start of the experiment. Chickens had access to a 1700 m² open-air range (3.4 m²/chicken) from 5 weeks of age, and were given commercial starter (1-13 j), grower (14-65 j) and finisher (66-77 j) diets formulated for slow-growing chickens (Vital Label, SCAR, Herve, Belgium). Cocks infected at 3-days-old by 10⁶ CFU *Campylobacter jejuni* strain LMG 8841 (Laboratorium voor Microbiologie, Gent, Belgium), and confirmed campylobacter-positive, were used to perform litter contamination by droppings before the setting up of the chicks and to contaminate the open-air range during one week before the exit of the chickens.

Each week, serum samples were collected from 20 chickens randomly selected in the broiler house for determination of anti-campylobacter IgG antibodies levels. These 20 chickens were then euthanized by an intracardiac dose of Nembutal[®] (Abbott Laboratories, North Chicago, IL) 2 mL/kg live weight, and bile (from 4 weeks of age) and cecal contents (from 1 week of age) were aseptically collected for *Campylobacter* enumeration and anti-campylobacter IgA quantification, respectively.

The counting method of *Campylobacter* in chicken caecal contents was based on direct plating on modified CampyCefex Agar (Hardy Diagnostics, Santa Maria, CA) containing 0.033 g/l cefepirone and 0.2 g/l cycloheximide. If no colony was recovered by direct plating, an enrichment step in Brucella broth (Biokar, Beauvais, France) and incubation 24 h at 42°C was performed. Total caecal content sample was diluted 10 folds with buffered peptone water and mixed thoroughly. The samples were serially diluted, and plated on modified CampyCefex agar. The plates were incubated at 42°C for 48 h in microaerophilic atmosphere generated in jars by using commercial gas generating kits (Anaerocult C, VWR International, Belgium). From each positive agar plate, several typical *Campylobacter* colonies were confirmed as a member of the genus by examination of cellular morphology and motility on a wet mount under phase contrast microscopy.

Campylobacter-specific IgG antibodies in serum and IgA antibodies in biliary content were measured by using an indirect enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with 100 µl/well of 2 µg/ml Outer Membrane Protein from *C. jejuni* (Virion/Serion, Würzburg, Germany) in coupling carbonate buffer (pH 9.6), overnight at 4°C. The wells were washed three times with ELISA wash (PBS + 0.2% v/v Tween 20) and then incubated with 250 µl/well of PBS containing 2% w/v bovine serum albumine for 30 min at room temperature. The wells were washed as before and then incubated with 100 µl chicken sera diluted (1:100 to 1:200,000) or chicken bile diluted (1:2 to 1:6,000) in ELISA diluent (PBS + 4% v/v Tween 20) for 1 h at room temperature with agitation. For detection, wells were washed 5 times as before and incubated with 100 µl goat anti-chicken IgG and IgA conjugated to peroxidase (Bethyl Laboratories, Montgomery, TX) diluted 1:10,000 in ELISA diluent for 1 h at room temperature with agitation. After washing, the bound peroxidase was detected by incubation with 100 µl of 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich, Bornem, Belgium) at room temperature with agitation. The reaction was stopped after 5 min by the addition of 100 µl/well 1 M H₂SO₄. The absorbance was read at 450 nm on a microplate titer. A four parameter logistic curve-fit was realised with standard serum or bile samples pooled from 3 *Campylobacter*-positive cocks used for environment contamination.

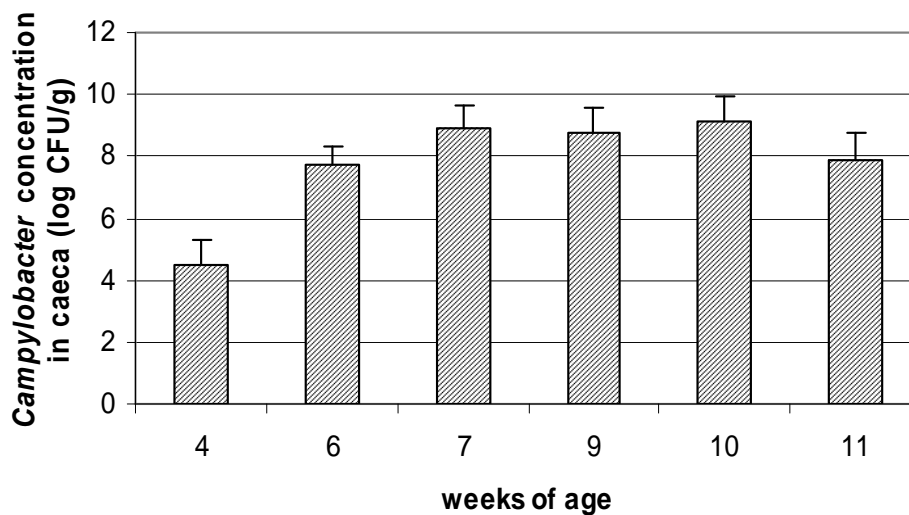
One-way analysis of variance was conducted with the general linear models procedure (GLM) of SAS (SAS Institute Inc., Cary, NC) and was followed by a Newman-Keuls test to calculate the significant differences in *Campylobacter* concentrations (log transformed) and antibodies titer values at each sampling point. Correlations between IgA titers and *Campylobacter* concentrations in the caeca according to the week of age were determined by the CORR

procedure of SAS with calculation of Pearson's linear correlation coefficient. For all statistical analysis, a *P* value of ≤ 0.05 was considered significant.

Results and discussion

The 1-day-old chicks were campylobacter-free before the start of the *in vivo* trial, as showed by the delivery tray analysis. *Campylobacter* concentrations in caeca of chickens from 1 to 11 weeks of age, reported in Figure 1, were recorded in order to investigate the natural flock infection by contaminated litter. The pathogen was for the first time detected in the flock at 4 weeks of age, in 30% (6/20) of the birds. One week later, all the 20 sampled chickens were campylobacter-positive. From 6 to 10 weeks of age, *Campylobacter* concentrations increased from 7.72 to 9.14 log CFU/g, but not significantly. On the other hand, the pathogen population showed a significant reduction at 11 weeks of age, with a mean concentration of 7.88 log CFU/g.

Figure 1. *Campylobacter* concentrations in caeca of broiler chickens from 4 to 11 weeks of age, after natural infection by straw litter contaminated by *C. jejuni* LMG 8841. Bars represent mean log cfu per gram of caecal content, and error bars indicate the SD. The values lacking a common letter are significantly different ($P < 0.05$).

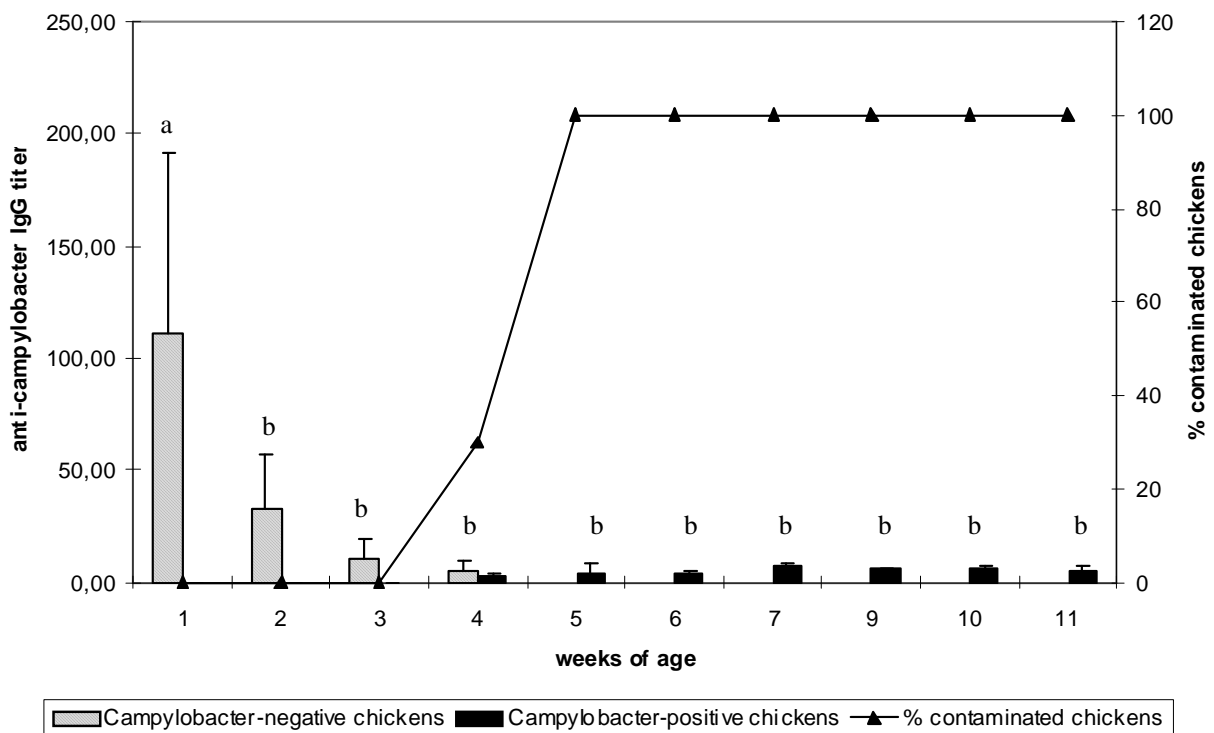


In organic and free-range broiler flocks, *Campylobacter* infection is generally more important than in conventional poultry production, with prevalence up to 100% (Newell and Fearnley, 2003; Vandeplas *et al.*, 2008). Once flock contamination is established, the majority of birds are colonized within only a few days, because of extremely rapid bird-to-bird transmission (Newell and Fearnley, 2003). Flock infection persists until the age of slaughter which may

consequently induce carcasses and poultry product contamination. Nevertheless, flock infection is generally a relatively late event. With efficient sanitary barriers inside the broiler house, the chickens may stay campylobacter-free until they have access to the open area (Rivoal *et al.*, 2005). Contamination may also appear before the chickens are in contact with the environment, but even in this case, most flocks become infected only 2 to 3 weeks after the placement of chicks (the so-called lag phase).

The natural infection of the chickens by *Campylobacter* was compared with immunological measurements. A high serum anti-campylobacter IgG titer of 110.13 was measured in one-day-old chicks. Afterwards, the IgG antibodies level showed a significant decrease to 5.60 for uninfected birds at 28 days of age (infected chickens IgG titer: 3.11, not significantly different from infected chickens), which coincided with the onset of *Campylobacter* infection in the flock, and reach a minimum of 3.39 at 5 weeks of age (Figure 2). From 5 to 11 weeks of age, IgG titer remained at relatively low levels, without significant differences according to the week of measurement.

Figure 2. Anti-campylobacter IgG antibodies levels in serum of broiler chickens reared on litter contaminated by *C. jejuni* LMG 8841 from 1-day-old, according to the week of age. Bars represent mean IgG titer based on a standard curve realised with serum samples from campylobacter-positive cocks, and error bars indicate the SD. The values lacking a common letter are significantly different ($P < 0.05$).



Sahin *et al.* (2001) stated that the appearance of *C. jejuni* infection may coincide with the disappearance of serum anti-campylobacter IgG during the first 3 to 4 weeks, as observed in this study. The immune system is immature in 1-day-old chick, and the functional maturation, which is a prerequisite for humoral response, occurs during the first two weeks of life (Bar-Shira *et al.*, 2003). Consequently, IgG detected during the post-hatch period may be maternal antibodies able to protect young chickens against colonization by *Campylobacter*. Once the functional immune competence is established, chicken colonization by *Campylobacter* induces a specific immune response with production of secretory antibodies in serum (Cawthraw *et al.*, 1994).

Results of IgA measurements of anti-campylobacter IgA titer in bile showed an increased immune response as birds aged. Secretory IgA titers were significantly different between 4 and 11 weeks of age (87 vs. 1146). Until 21 days, specific IgA levels were too low to be detected. This local specific antibody response localised in the bile was showed to be negatively correlated with the *Campylobacter* concentrations in caeca during all the experimental period (Table 1), with the highest IgA titer observed at 11 weeks corresponding to the significant reduction of *C. jejuni* contamination.

In chickens, the Gut-Associated Lymphoid tissue (GALT) is responsible for inducing immune responses against bacterial, viral and parasitic enteral antigens. The increased anti-campylobacter IgA concentrations as the birds become older are provided by the GALT maturation as the immune system develops (Bar-Shira *et al.*, 2003). IgA antibodies may be secreted by the plasmocytes of the lamina propria, which is one of the lymphoid structures of the GALT. IgA are then found in high concentrations in the intestine and in the bile (Mockett, 1986). Cawthraw *et al.* (1994) suggested that increased serum IgA concentration is in correlation with the reduction of *Campylobacter* colonization. According to Edens (2003), elevated levels of IgA may be associated with increased rate of bacterial clearance via antibody-mediated phagocytosis.

Table 1. Anti-campylobacter IgA antibodies levels in bile of broiler chickens reared on litter contaminated by *C. jejuni* LMG 8841 from 4 weeks of age, and correlation with *Campylobacter* concentrations in the caeca, according to the week of age.

	Weeks of age							SEM ¹
	4	5	6	7	9	10	11	
Anti-campylobacter IgA (titer)	^a 87	^{ab} 270	^{ab} 747	^{ab} 799	^b 1298	^{ab} 713	^b 1146	126.5
<i>Campylobacter</i> concentration in caeca (log CFU/g \pm SD ¹)	^a 4.48 \pm 0.84	nd	^b 7.72 \pm 0.60	^c 8.88 \pm 0.80	^{bc} 8.74 \pm 0.81	^{bc} 9.14 \pm 0.80	^d 7.88 \pm 0.91	0.283
Correlation coefficient	-0.2220	nd	-0.5243	-0.6121	0.6722	-0.5439	0.7076	
<i>P</i> value	0.7780	nd	0.0042	0.0200	0.0085	0.0675	0.01	

^{a-d} Means within a row lacking a common superscript are significantly different ($P < 0.05$).

nd: not determined

¹ SD: standard deviation; SEM: standard error of the mean

It would be further interesting to follow serum antibody levels of uninfected chickens. However, broiler flocks in contact with the outer environment are frequently infected by *Campylobacter*, as described by Vandeplass *et al.* (2008), and keeping *Campylobacter*-free chickens in rearing conditions similar to these of the present study could be rather difficult. Moreover, uninfected animals only produce a low and constant specific immune response against the pathogen (Cawthraw *et al.*, 1994).

In summary, this study showed that infection of a slow-growing broiler flock reared from 1-day-old on straw litter naturally contaminated by *C. jejuni* became infected only from 3-4 weeks of age and that *Campylobacter* contamination persisted until the age of slaughter. These results may demonstrate a protective role of campylobacter-specific maternal IgG antibodies with high serum levels detected in 1-day-old chicks. At 4 weeks of age, the titer was reduced 16 times. At the same time, the immune system progressively develops, and lymphoid structures of the avian GALT mature, with increased production of antibodies directed toward enteral antigens. Anti-campylobacter IgG levels showed little increase in the serum from 4 to 11 weeks of age, whereas specific biliary IgA titers rose significantly. Moreover, IgA levels were negatively correlated with *Campylobacter* concentrations measured in the chicken caeca during all the rearing period. The increase of secretory immune response as bird age may help to limit *Campylobacter* population in the chicken gut.

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