Campylobacter jejuni is not merely a commensal in commercial broiler chickens and affects bird welfare

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Campylobacter jejuni Is Not Merely a Commensal in Commercial Broiler Chickens and Affects Bird Welfare

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ABSTRACT Campylobacter jejuni is the leading cause of bacterial food-borne infection; chicken meat is its main source. C. jejuni is considered commensal in chickens based on experimental models unrepresentative of commercial production. Here we show that the paradigm of Campylobacter commensalism in the chicken is flawed. Through experimental infection of four commercial breeds of broiler chickens, we show that breed has a significant effect on C. jejuni infection and the immune response of the animals, although these factors have limited impact on the number of bacteria in chicken ceca. All breeds mounted an innate immune response. In some breeds, this response declined when interleukin-10 was expressed, consistent with regulation of the intestinal inflammatory response, and these birds remained healthy. In another breed, there was a prolonged inflammatory response, evidence of damage to gut mucosa, and diarrhea. We show that bird type has a major impact on infection biology of C. jejuni. In some breeds, infection leads to disease, and the bacterium cannot be considered a harmless commensal. These findings have implications for the welfare of chickens in commercial production where C. jejuni infection is a persistent problem.

IMPORTANCE Campylobacter jejuni is the most common cause of food-borne bacterial diarrheal disease in the developed world. Chicken is the most common source of infection. C. jejuni infection of chickens had previously not been considered to cause disease, and it was thought that C. jejuni was part of the normal microbiota of birds. In this work, we show that modern rapidly growing chicken breeds used in intensive production systems have a strong inflammatory response to C. jejuni infection that can lead to diarrhea, which, in turn, leads to damage to the feet and legs on the birds due to standing on wet litter. The response and level of disease varied between breeds and is related to regulation of the inflammatory immune response. These findings challenge the paradigm that C. jejuni is a harmless commensal of chickens and that C. jejuni infection may have substantial impact on animal health and welfare in intensive poultry production.
systemic specific antibodies (7–9). Studies of T lymphocyte function in the gastrointestinal tract during C. jejuni infection are very limited, although T cell responses have been shown in the liver during invasive infections (10). One potential criticism of the past work is that many studies used specific-pathogen-free (SPF) chickens with high challenge doses that have only limited relevance to infection and immunity in commercial broiler breeds. Modern broilers, as exemplified by the A1 and A2 and the C line birds tested in this study, have been bred to grow rapidly and to convert feed into muscle tissue efficiently while keeping high conformity of size and shape to facilitate slaughter and production processes. Such birds reach slaughter weights of approximately 2.2 kg at ~36 days of age. The downside of selective breeding in order to achieve the above production goals are several welfare-associated conditions in these birds, ranging from musculoskeletal disorders to gastrointestinal problems. In contrast, traditional breeds such as the White Leghorn and Light Sussex used in SPF systems where stocking densities of up to 40 kg/m^2 are employed. The data for the birds are summarized in Materials and Methods.

### RESULTS

**Broiler breed has little impact on C. jejuni colonization of the ceca following experimental infection.** Broiler breed had no significant impact on C. jejuni M1 levels in ceca following experimental infection of broiler chickens (Table 1), nor was there a statistically significant (P = 0.068) impact on C. jejuni numbers in this gut compartment at slaughter age (Table 2). Performance data for the birds are summarized in Materials and Methods.

**C. jejuni elicits early innate immune activation and an inflammatory response in broilers, which remains elevated for longer in the fast-growing breed A1.** The innate immune responses to C. jejuni were examined in detail in birds from the A1, A2, B1, and B2 lines. Infection elicited expression of the pro-inflammatory chemokines CXCLi1 (chemokine [C-X-C] motif ligand 1) (Fig. 1a.i), CXCLi2 (Fig. 1b.i), and interleukin-1β (IL-1β) (Fig. 1c.i) in the ceca of all breeds at 2 days postinfection (dpi). CXCLi2 expression was also detected in the ileum (Fig. 2a). By 5 dpi, these responses were reduced (Fig. 1a.ii, b.ii, and c.ii) with the exception of the A1 birds in which the levels of expression of all three inflammatory mediators remained significantly higher than in the other birds (CXCLi1 [P = 0.017], CXCLi2 [P < 0.000], and IL-1β [P = 0.012]). By 12 dpi, expression of CXCLi2 remained higher in the C. jejuni-infected A1 group than in the other bird types (A2 [P = 0.027] and B2 [P = 0.019]), although IL-1β expression was not statistically different.

### Table 2

| Breeding line | Slaughter age (days) | Median C. jejuni M1 cecal content (CFU · g⁻¹) [range] |
|---------------|---------------------|-----------------------------------------------------|
| A1            | 35                  | 2.88 × 10⁸ [4.58 × 10⁷−1.93 × 10⁹]                  |
| C             | 36                  | 3.84 × 10⁸ [8.78 × 10⁷−6.86 × 10⁸]                  |
| A2            | 39                  | 4.94 × 10⁹ [3.08 × 10⁸−2.00 × 10⁹]                  |
| B1            | 48                  | 5.30 × 10⁸ [3.38 × 10⁷−2.00 × 10⁸]                  |
| B2            | 56                  | 1.22 × 10⁸ [1.49 × 10⁷−5.77 × 10⁸]                  |
pression was reduced (Fig. 1b.iii and c.iii). All four bird breeds showed expression of CXCL1 at 12 dpi, but with no significant differences ($P = 0.339$) between them (Fig. 1a.iii).

These findings show that the four breeds tested express key inflammatory signals in the innate immune system early after oral infection with $C. jejuni$, but these inflammatory signals are elevated in the A1 birds for much longer. There were no differences in basal constitutive expression of these genes in uninfected control birds of each breed. Considerable variation could be seen in the response between individuals within groups. This is perhaps not surprising, given that these are outbred commercial animals with inherent genetic differences between individuals, although the use of a relatively large group size reduces the impact of such variation.

Histological examination of the ilea and ceca of uninfected birds of all breeds identified an apparently constitutive mild diffuse lymphoplasmacellular mucosal infiltration (Fig. 3A, B, E and G). Following infection of the fastest (A1) and slowest (B2) growing birds, increased numbers of inflammatory cells, heterophils, and lymphocytes were seen (Fig. 3C, D, F, and H), commensurate with the cytokine responses seen at 5 dpi. In both breeds, there was evidence of mucosal damage, notably thickening, shortening, and fusion of villi in the ileum (Fig. 3C and D). This was more frequently observed and more intense in the A1 breed where such pathological changes were seen in 7 of 10 birds. In contrast, only 2 of 10 infected B1 bird and 2 of 9 infected A2 birds had pododermatitis. This condition was not seen in the infected B2 group.

Disease in some broiler breeds is a consequence of immune dysregulation in the intestine. Differential expression of IL-10 in breeds was seen in the ceca at 12 dpi (Fig. 4a) with a median 50-fold increase in expression in the slower-growing B2 birds, significantly higher than the minimal levels in the A1 animals ($P = 0.003$) (see Fig. S1 in the supplemental material). Expression in the A2 and B1 birds was intermediate between these extremes. IL-10 is a key regulatory cytokine in controlling inflammation. The absence of IL-10 expression in the A1 birds indicates that inflammatory responses in the intestine are poorly regulated, leading to prolonged inflammation and diarrhea, as described above. A sim-

Prolonged inflammation in the intestines of faster-growing breeds of chickens results in diarrhea and affects their welfare. By 12 dpi, the infected A1 birds were found to have diarrhea when examined during twice-daily welfare checks and at ante mortem examination where fecal pasting of feathers around the cloaca consistent with diarrhea was found. Similar observations were also made in C birds infected to examine longer-term carriage. Although feces were generally wetter in A1 birds than in other breeds, no diarrhea was seen in the control group. Diarrhea was not seen in the A2 or B birds. At postmortem examination, A1 birds were found to have more watery intestinal contents and some evidence of inflammation, such as mild hyperemia of the cecal wall. Diarrhea in the A1 birds corresponds to the greater and prolonged inflammatory response seen in this breed. Furthermore, despite birds being maintained under experimental conditions, including regular changes of litter, 9 of 10 $C. jejuni$-infected A1 birds showed signs of pododermatitis at 12 dpi. In contrast, only 2 of 10 infected B1 bird and 2 of 9 infected A2 birds had pododermatitis. This condition was not seen in the infected B2 group.

FIG 1 Expression of proinflammatory cytokines and chemokines in cecal tissue following $C. jejuni$ M1 infection. (a to c) Fold changes in expression of chemokines CXCL1 (a) and CXCL2 (b) and cytokine IL-1β (c) in cecal tissue were examined at 2 (i), 5 (ii), and 12 (iii) dpi. At each time point, group sizes were as follows for the different breeds: $n = 10$ for breed A1, $n = 9$ for breed A2, $n = 10$ for breed B1, and $n = 10$ for breed B2. Each symbol represents the value for an individual chicken. A white bar represents the median value for each group. Significance of differences between the groups was examined using a Kruskal-Wallis test.
an inflammatory innate immune response in the intestinal tract, which led to a specific antibody response. Intestinal disease in the A1 birds appears to be a consequence of a poorly regulated immune response, as expression levels of proinflammatory signals such as CXCL chemokines and IL-1β remain higher than in the three other breeds at 5 and 12 dpi. In contrast, IL-10 expression is significantly higher in B2 birds at 12 dpi, suggesting that it is much better able to regulate its inflammatory response and that dysregulation of this in A1 birds leads to infection-mediated pathology in the lower intestinal tract. The importance of IL-10 in regulating C. jejuni enteritis has been shown in the C57BL/6 IL−10−/− mice used as model for acute enterocolitis (15, 16). While both control and IL-10-deficient C57BL/6 mice can be colonized by C. jejuni, only C57BL/6 IL−10−/− mice develop enteritis (17).

The data presented here show that there is no intrinsic difference in the susceptibility of broiler breeds to C. jejuni under experimental conditions. As such, it is unclear as to whether any change in the current broiler breed type will have any impact on public health. However, breeding chickens resistant to Campylobacter infection is considered, along with vaccination, to be a potential long-term intervention in controlling these bacteria in chicken production (18, 19). Studies using inbred and outbred lines of chicken have indicated that differences in innate immunity, particularly β-defensins secreted in the gut, may hold the key to resistance (20). However, more-detailed transcriptional studies of the response to C. jejuni in two broiler lines differing in resistance to colonization indicate that there is greater activity of regulatory T lymphocytes in the more resistant line (20, 21). T regulatory cells (or TRegs) are important in maintaining gut homeostasis and regulating the response to infection of the chicken and other species through production of cytokines such as IL-10 and tumor growth factor beta (TGF-β). Taken with our data on expression of IL-10, it appears that regulation of responses through T regulatory lymphocytes is key to both maintaining a healthy gut and reducing colonization by C. jejuni following infection. As such, we suggest that certain faster-growing breeds of broiler chicken, such as the A1 breed, have a functional innate response to Campylobacter infection but do not have an effective T regulatory one, leading to the pathological changes we have seen. This dysfunction, if it occurs with other enteric pathogens, may also lead to inflammatory damage in the intestinal tract under production conditions. In our past work (11, 12), we did not ascertain whether pododermatitis and hock marks, linked to an increased risk of Campylobacter infection, preceded infection or occurred because of it. Data presented here show that C. jejuni makes a direct contribution to this condition. The immune dysfunction we have seen in response to C. jejuni does not seem to be linked to more rapid growth per se and is more likely to be related to the genetic background of the birds, as this will differ even in animals bred by the same company. Such information is commercially sensitive and therefore not freely available.

FIG 2 Expression of proinflammatory chemokine CXCL12 in ileal tissue following C. jejuni M1 infection. (a to c) Fold changes in expression of CXCL12 in ileal tissue were examined at 2 (a), 5 (b), and 12 (c) dpi. At each time point, group sizes were as follows for the different breeds: n = 10 for breed A1, n = 9 for breed A2, n = 10 for breed B1, and n = 10 for breed B2. Each symbol represents the value for an individual chicken. A white bar represents the median value for each group. Significance of differences between the groups was examined using a Kruskal-Wallis test.

In summary, we show that C. jejuni is not merely a harmless gut commensal of some modern commercial broiler chickens. Infection of broiler chickens is clearly associated with intestinal inflammation. While the possibility of this response involving some interaction with other bacteria in the intestinal microbiota cannot be ruled out, it is clear that the infection with C. jejuni is essential in causing the inflammation described. In the fast-growing A1 breed, this inflammation is prolonged, leading to diarrhea and subsequent associated pathology to the feet and legs of these birds. Diarrhea was also seen in breed C birds, but detailed innate immune responses in this breed were not examined because of logistical constraints. Detailed examination of responses to infection in four broiler breeds found that C. jejuni M1 elicited a direct contribution to this condition. The immune dysfunction we have seen in response to C. jejuni is centered upon such as CXCL chemokines and IL-1α which led to a specific antibody response. Intestinal disease in the A1 birds appears to be a consequence of a poorly regulated immune response, as expression levels of proinflammatory signals such as CXCL chemokines and IL-1β remain higher than in the three other breeds at 5 and 12 dpi. In contrast, IL-10 expression is significantly higher in B2 birds at 12 dpi, suggesting that it is much better able to regulate its inflammatory response and that dysregulation of this in A1 birds leads to infection-mediated pathology in the lower intestinal tract. The importance of IL-10 in regulating C. jejuni enteritis has been shown in the C57BL/6 IL−10−/− mice used as model for acute enterocolitis (15, 16). While both control and IL-10-deficient C57BL/6 mice can be colonized by C. jejuni, only C57BL/6 IL−10−/− mice develop enteritis (17).

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confining the pathogen to the intestine where it persists and that the response in the gut is tightly regulated to prevent excessive inflammation, which in traditional chicken breeds leads to little or no ill effect on the bird. However, in modern broiler breeds, the drive toward improved productivity and carcass conformity would appear to have had a negative impact on T lymphocyte function, leading to a loss of immune regulation in the gut. The result of this imbalance is that *C. jejuni* infection results in poorly controlled inflammation, leading to intestinal mucosal damage and diarrhea.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *C. jejuni* M1 was kindly provided by Lisa Williams (University of Bristol). Bacteria were grown from stocks maintained at $-80^\circ$C on Columbia blood agar (Lab M, Heywood, Lancashire, United Kingdom) supplemented with 5% defibrinated horse...
Liverpool high-biosecurity poultry unit. Chicks were maintained in floor from a commercial hatchery. Chicks were housed in the University of experiments, and the decision to use breed A birds for detailed examination was approved by the University of Liverpool ethical review process prior to the award of the licenses. All animals were checked a minimum of twice to limit retransmission within groups and to reduce problems associated with wet litter.

Effect of broiler breed on *C. jejuni* infection (experiment 1). When the chicks were 21 days old, A1 (n = 40), A2 (n = 38), B1 (n = 40), and B2 (n = 40) broilers were orally infected with 10^5 cells of *C. jejuni* M1 in 0.2 ml of MHB. Control birds (A1 [n = 16], A2 [n = 15], B1 [n = 15], and B2 [n = 16]) received 0.2 ml of sterile MHB. At 2, 5, and 12 dpi, 10 infected birds and 5 control birds of each breed were killed by cervical dislocation. At postmortem examination, samples of tissue and gut contents were taken and processed for host gene expression analysis, histology, and *Campylobacter* enumeration.

Assessment of *C. jejuni* load. In order to determine the levels of *C. jejuni* colonization in each of the breeds, cecal contents were collected from individual birds at necropsy and diluted in 9 volumes of maximal recovery diluent (MRD). Serial 10-fold dilutions were made of each sample in MRD, and using the method of Miles et al. (22), duplicate 50-μl spots were plated onto mCCDA agar supplemented with SV59. The plates were incubated under microaerobic conditions at 41.5°C for 48 h, and *Campylobacter* colonies were enumerated to give CFU/g of cecal contents.

RNA extraction and real-time qRT-PCR. Samples of ileal and cecal tissue from infected and control birds were collected and stored in 500 μl of RNAlater at −20°C (Sigma, Poole, Dorset, United Kingdom). Total RNA was isolated from 20 to 30 mg of tissue using an RNeasy minikit (Qiagen, West Sussex, United Kingdom) according to the manufacturer’s instructions. Isolated RNA was eluted into 50 μl RNease-free water and stored at −80°C. The yield of total RNA was determined using a NanoDrop (ND-1000) spectrophotometer.

Expression of mRNA for the cytokines interleukin-1β (IL-1β), gamma interferon (IFN-γ), IL-10, and IL-4 and the chemokines CXCL1 (chemokine [C-X-C motif] ligand 1) and CXCL2 in these tissues was measured by real-time quantitative reverse transcription-PCR (qRT-PCR), using primer and probe sequences described by Shini and Kaiser (23). 28S RNA was used as the housekeeping gene. Twenty-microliter reaction mixtures for one-step qRT-PCR were prepared using theRotorGene Probe RT-PCR kit (Qiagen, West Sussex, United Kingdom) and contained 1 μl of total RNA (at 20 ng/μl), 10 μl of RotorGene Probe RT-PCR master mix, 0.2 μl of RotorGene reverse transcriptase enzyme mix, 1.6 μl of each primer (at 10 μM), 0.8 μl of probe (at 5 μM), and 4.8 μl of RNease-free water. Triplicate reactions were set up for each sample using an automated QIAbility system (Qiagen, West Sussex, United Kingdom). Real-time RT-PCR was performed on a RotorGene Q system (Qiagen, West Sussex, United Kingdom) with the following reaction profile: 50°C for 10 min to facilitate reverse transcription of RNA to cDNA, followed by 95°C for 5 min, and 40 repeat cycles, with 1 cycle consisting of 95°C for 5 s and 60°C for 10 s.

Expression of the target gene was determined using the cycle threshold (CT) value relative to that for the 28S rRNA reference gene (ΔCT). Results are expressed as fold changes in corrected target gene expression (ΔΔCT) in infected animals relative to the control animals (2−ΔΔCT).

Histology. Samples of ileum and cecum from the two breeds displaying the most extreme differences in cytokine response in the experiments, A1 and B2, were examined for histological assessment. These samples were obtained from the same infected and uninfected birds used for cytokine analysis. Tissue samples were fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) and routinely embedded in paraffin wax. Sections (3 to 5 μm thick) were prepared and stained with hematoxylin and eosin (H&E) using standard protocols. Tissue sections were assessed in a blind manner by veterinary pathologists for any pathological changes and the semiquantitative analysis of the inflammatory infiltrate, represented by grading the degree of heterophil and lymphoplasmacellular infiltration as follows: 1 for slight infiltration, 2 for mild to moderate infiltration, 3 for marked infiltration, and 4 for severe infiltration.
infiltration, 3 for moderate infiltration, 4 for marked infiltration, and 5 for severe infiltration.

C. jejuni carriage at slaughter age (experiment 2). When the chicks were 21 days old, A1 (n = 7), C (n = 9), A2 (n = 9), B1 (n = 9), and B2 (n = 10) birds were orally infected with 10^5 cells of C. jejuni M1 in 0.2 ml of MHB. The birds were grown to their commercial slaughter ages (36 days for A1 breed, 36 days for C, 37 days for A2, 48 days for B1, and 56 days for B2) and were killed by cervical dislocation. At postmortem examination, the contents were taken from both ceca for Campylobacter enumeration using the method described above. Original groups contained 10 birds, but because of welfare concerns unrelated to C. jejuni infection, some birds were culled prior to the end of the experiment.

Statistical analyses. Statistical analyses were performed using SPSS v20 (IBM). For comparison of bacterial loads between infected groups and fold changes in cytokine expression between breeds, the Kruskal-Wallis (nonparametric analysis of variance [ANOVA]) test was applied, as the data were not normally distributed. Differences were considered significant where P < 0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01364-14/-/DCSupplemental.

Figure S1, TIF file, 4.4 MB.
Figure S2, TIF file, 4.4 MB.
Figure S3, TIF file, 4.4 MB.

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