IMMUNOLOGY, HEALTH, AND DISEASE

Campylobacter jejuni colonization promotes the translocation of Escherichia coli to extra-intestinal organs and disturbs the short-chain fatty acids profiles in the chicken gut

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ABSTRACT For a long time Campylobacter was only considered as a commensal microorganism in avian hosts restricted to the ceca, without any pathogenic features. The precise reasons for the symptomless chicken carriers are still unknown, but investigations of the gastrointestinal ecology of broiler chickens may improve our understanding of the microbial interactions with the host. Therefore, the current studies were conducted to investigate the effects of Campylobacter jejuni colonization on Escherichia coli translocation and on the metabolic end products (short-chain fatty acids, SC-FA). Following oral infection of 14 day old broiler chickens with 1 × 10⁸ CFU of Campylobacter jejuni NCTC 12744 in two independent animal trials, it was found that C. jejuni heavily colonized the intestine and disseminate to extra-intestinal organs. Moreover, in both animal trials, the findings revealed that C. jejuni promoted the translocation of E. coli with a higher number encountered in the spleen and liver at 14 days post infection (dpi). In addition, Campylobacter affected the microbial fermentation in the gastrointestinal tract of broilers by reducing the amount of propionate, isovalerate, and isobutyrate in the cecal digesta of the infected birds at 2 dpi and, at 7 and 14 dpi, butyrate, isobutyrate, and isovalerate were also decreased. However, in the jejunum, the C. jejuni infection lowered only butyrate concentrations at 14 dpi. These data indicated that C. jejuni may utilize SC-FA as carbon sources to promote its colonization in the chicken gut, suggesting that Campylobacter cannot only alter gut colonization dynamics but might also influence physiological processes due to altered microbial metabolite profiles.

Finally, the results demonstrated that C. jejuni can cross the intestinal epithelial barrier and facilitates the translocation of Campylobacter itself as well as of other enteric microorganisms such as E. coli to extra-intestinal organs of infected birds. Altogether, our findings suggest that the Campylobacter carrier state in chicken is characterised by multiple changes in the intestinal barrier function, which supports multiplication and survival within the host.

Key words: Campylobacter jejuni, broiler chickens, intestinal colonization, Escherichia coli translocation, microbial fermentation

INTRODUCTION

Campylobacter is the most important food borne pathogen, primarily associated with poultry (EFSA, 2011). The bacterial colonization undeniably requires a capacity to respond to environmental changes and to survive the hazardous conditions within the gastrointestinal tract. Thus, new information concerning how C. jejuni colonizes poultry is important in developing strategies aimed at reducing or eliminating C. jejuni carriage.

The epithelial barrier serves as an infectious blockade for many bacterial pathogens but is also an important entry port for pathogens which might disseminate into internal organs (Awad et al., 2012, 2014a,b). In contrast to the general perception that C. jejuni is only a commensal in chickens, it was reported that Campylobacter is able to invade the chicken’s intestinal mucosa with further spread to internal organs (Lamb-Rosteski et al., 2008; Van Deun et al., 2008; Weber et al., 2014). Furthermore, it was demonstrated that C. jejuni colonization in the chicken intestine was accompanied by mucosal damage and a higher intestinal permeability which
suggests that \textit{C. jejuni} translocates via the paracellular and the transcellular pathway (Humphrey et al., 2014; Awad et al., 2014a, 2015a,b). Recently, we also revealed that \textit{Campylobacter} interacts intimately with the gut epithelium and influences the host cellular functions by interfering with Ca$^{2+}$ signaling and nutrient absorption (Awad et al., 2015b). Consequently, the ability of \textit{Campylobacter} to colonize the chicken gut is multifactorial and many factors are involved in this process.

\textit{Campylobacter} requires numerous virulence factors to successfully colonize the host, to translocate, and to avoid clearance by the host immune system (Ketley 1997). Furthermore, \textit{Campylobacter} colonization is regulated by environmental factors, e.g., changes in nutrient availability, osmolarity, pH, and organic acid concentrations which can alter its virulence (Sun and O’Riordan, 2013). Additionally, the capability of \textit{Campylobacter} to translocate across the intestinal barrier is considered to be an important virulence feature, which allows the bacterium’s access to underlying tissues and could promote the dissemination throughout the host (Konkel et al., 2001).

The gastro-intestinal tract of chickens harbors numerous bacterial species that play a vital role in the normal physiological, immunological, and protective functions of the host (Rehman et al., 2007). It is known that bacterial fermentation in the gut leads to the formation of short-chain fatty acids (SCFAs), an important source of energy for enterocytes and they are vital for intestinal health (Sunkara et al., 2012). Furthermore, increased concentrations of SCFAs lower the intestinal pH, which is associated with a suppression of pathogens (Kubena et al., 2001, Rehman et al., 2007). Thus, SCFAs are of particular importance and have been used frequently to assess the bacterial metabolism in the intestine. SCFAs not only affect host functions but also serve as a carbon source for the endogenous bacteria and at high concentration can exhibit toxic effects on bacteria. Sun and O’Riordan (2013) reported that \textit{C. jejuni} may be better adapted to utilize SCFA as a source of carbon and energy in the intestines because of its inability to utilize carbohydrates.

Generally, gut microbiota modulate host responses to limit the colonization of pathogens. Recently, it was shown that \textit{C. jejuni} colonization is dependent on the microflora of the specific host and vice versa that \textit{Campylobacter} colonization induces a change in the intestinal microbiota (Haag et al., 2012; Sofka et al., 2015). Following colonization, intestinal epithelial barrier disruption may contribute to the translocation of \textit{Campylobacter} itself and other luminal bacteria which are relatively unknown thus far. As a consequence of this it can be hypothesized that \textit{C. jejuni} colonization affects metabolic end products derived from the intestinal microbiota in chickens. Consequently, the objectives of this study were: (1) to determine whether \textit{Campylobacter} colonization promotes the translocation of \textit{E. coli}; and (2) to delineate the effects of a \textit{C. jejuni} infection on metabolic end products derived from the intestinal microbiota.

**MATERIALS AND METHODS**

**Ethics Statement**

Animal experiments were approved by the institutional ethics committee of the University of Veterinary Medicine and the Ministry of Research and Science under the license number GZ 68.205/0011-11/3b/2013. All husbandry practices were performed with full consideration of animal welfare.

**Bacterial Strains, Media and Growth Conditions**

\textit{C. jejuni} reference strain NCTC 12744 was routinely cultivated at 42°C for 48 h under microaerophilic conditions (5% O2, 10% CO2, 85% N2) on Campylosel agar (BioMerieux, Vienna, Austria) or modified charcoal-cefaprazone-deoxycholate (mCCD) agar (CM0739, OXOID, Hampshire, UK). \textit{Escherichia coli} was routinely grown at 37°C for 24 h on MacConkey agar (Scharlau, Barcelona, Spain).

**Experimental Design**

Two animal trials consisting of two groups each with an identical set up were performed. In both successively performed trials commercial broiler chickens (Ross-308, Geflügelhof Schulz, Graz, Austria) obtained from the same hatchery were used. The birds were housed on wood shavings with feed and water provided ad libitum.

In the first study 40-day-old birds were split equally in two groups. In the second trial the animal numbers were increased to 60 birds per group. In each trial birds in one group were infected with \textit{Campylobacter jejuni} reference strain NCTC 12744 and the second group was kept as control being non-infected and inoculated with PBS only.

At 1 day of age and again prior to infection, birds were confirmed as \textit{Campylobacter}-free by taking cloacal swabs which were streaked onto mCCD agar (CM0739, OXOID, Hampshire, UK) and grown for 48 h under microaerophilic conditions at 42°C. The infection was performed orally via feeding tube (gavage) at a dose of $1 \times 10^8$ CFU/bird at 14 days of age as previously described (Awad et al., 2015a). Until the end of animal trials, the birds were monitored daily for any clinical signs.

At different days post infection (dpi), in both animal trials, ten birds from each group were anaesthetized by injection of a single dose of thiopental (20 mg/kg) into the wing vein and killed by bleeding of the jugular vein, gastrointestinal contents from the jejunum and ceca, and liver and spleen tissues were taken for \textit{C. jejuni} or \textit{E. coli} enumeration using the method described below. In addition, at 2, 7, and 14 dpi, cloacal swabs were collected for determining the shedding pattern of \textit{C jejuni} in broiler chickens. In the second animal trial, in order to better understand other potential underlying reasons for high \textit{Campylobacter} colonization load in chickens,
the jejunal and cecal contents from ten birds were immediately frozen (−80°C) until analysis for measuring the short-chain fatty acid concentrations.

**Bacterial Enumeration**

For *C. jejuni* or *E. coli* detection in the intestine (jejunal and cecal contents), liver and spleen, 1 g from each segment was collected from ten birds per group at each time point (7 and 14 dpi) at necropsy, and diluted 1:10 (wt:vol) in phosphate-buffered saline (PBS) (BR0014G, OXOID, Hampshire, UK) in both animal trials. The mixture was homogenized using an Ultra-Turrax (IKA, Staufen, Germany). Afterwards, serial 10-fold dilutions were made from each sample, 100 μL from each dilution were direct-plated in duplicate onto Campylosel agar (BioMerieux, Vienna, Austria). The plates were incubated at 37°C for 48 h, and typical *Campylobacter* colonies were enumerated as colony-forming units per gram. Furthermore, for *E. coli*, 100 μL from each dilution was direct-plated in duplicate on MacConkey agar (Scharlau, Barcelona, Spain). The plates were incubated at 37°C for 24 h. After incubation, *E. coli* colonies were counted as colony-forming units per gram.

**Short-Chain Fatty Acids (SCFAs) Analysis**

From ten birds per group euthanized at 2, 7, and 14 dpi in the second trial, the digesta of the jejunum and cecum was gently flushed out and frozen at −80°C until analysis. After thawing, the digesta samples were diluted with distilled water (1:5). Following homogenization, the samples were centrifuged at 6000 × g for 5 minutes. The supernatant (500 μL) was transferred into a tube containing 500 μL of ether. The samples were again centrifuged at 10000 × g for 5 minutes and the supernatant was then analyzed with a gas chromatograph. The SCFAs analyses were done as described by Atteh et al. (2008). A standard SCFAs mixture (20 mmol/L) of acetic, propionic, isobutyric, butyric, isovaleric, valeric acid was used for calibration as external standard. One microliter of the ether phase extract was injected into a Gas Chromatograph (TRACE 2000, Thermo Scientific). The column was a Nukol Fused Silica Capillary Column 30 m × 0.25 mm with 0.25 μm film thickness (Supelco). Helium was used as carrier gas at a pressure of 83 kPa. The detector type was FID with a split injector (1:50). Injection and detection temperatures were 220 and 250°C, respectively.

**Statistical Analysis**

Data are presented as means with SEM. Following tests for normality (Kolmogorov-Smirnov’s test), statistical analysis of SCFAs and bacterial translocation data for significant differences between the two groups was performed using Student *t* test. All tests were performed using PASW statistics 20, SPSS software (Chicago, IL).

**RESULTS**

**Campylobacter jejuni Colonization of Intestine and Internal Organs**

In both animal trials, no *Campylobacter* were detected in swab samples taken from day-old birds and prior to infection. Non-infected birds stayed *Campylobacter*-negative throughout the experiment and in both animal trials, no clinical signs were observed after oral infection with *Campylobacter*. Fecal droppings looked normal in both control and infected birds, no signs of diarrhea and no mortality occurred over the course of experiments. Shedding of *Campylobacter* could be confirmed at 2 dpi in both trials. *C. jejuni* was detected in the jejunum, ceca, liver, and spleen of the infected birds at 7 and 14 dpi, determined by bacterial plating.

In the first trial, *C. jejuni* was detected in the jejunum, cecum, liver, and spleen of the infected birds. The colonization was higher (*P* < 0.05) in the jejunum (2.16 ± 0.46 × 10^8 CFU/g) and cecum (1.13 ± 0.13 × 10^14 CFU/g) at 14 dpi compared to the colonization at 7 dpi in the jejunum (0.33 ± 0.29 × 10^6 CFU/g) and cecum (1.12 ± 0.24 × 10^10 CFU/g), respectively. Furthermore, the numbers of *C. jejuni* in the liver and spleen reached to 2.92 ± 1.93 × 10^4 and 0.55 ± 0.09 × 10^4 CFU/g, respectively, at 14 dpi. The results revealed that the colonization persisted for the duration of the experiment with a maximal bacterial load in the ceca at 14 dpi. In the second trial, very similar data were obtained with highest bacterial numbers in the cecum at 14 dpi and spread of *C. jejuni* to the liver and spleen. Such data were reported recently in connection with the influence of *C. jejuni* on glucose uptake and Ca^{2+} signaling and are published in a separate paper (Awad et al., 2015b). The results of both trials confirmed that the maximal bacterial load was observed in the cecum at 14 dpi. Re-cultivation of *C. jejuni* from the liver and spleen demonstrated its ability to spread to extra-intestinal organs.

**Influence of Campylobacter Infection on the Translocation of *E. coli***

The *E. coli* enumeration in the digesta content of the small and large intestine in both trials is presented in Figure 1. In the first trial, birds had lower *E. coli* loads in the jejunum and cecum at 7 dpi and in cecum at 14 dpi compared to the controls. In contrast, *E. coli* counts were higher in the liver and spleen of *C. jejuni* infected birds at 7 dpi and 14 dpi compared to the controls. These data demonstrate that *E. coli* translocation increased to the liver and spleen of the infected birds. The results of *E. coli* translocation in the second trial were
Figure 1. C. jejuni-induced translocation of E. coli to liver (Li) and spleen (Sp) of infected birds, whereas, E. coli counts in the jejunal (Je) and cecal (Ce) contents were reduced in trial 1 (A) and trial 2 (B) at different times post infection. Results are presented as means values and SEM (n = 10). Numbers of bacteria are expressed in logarithmic form of colony forming units (log CFU/g). Asterisks mark differences to resp. control with *P < 0.1 (*), **P < 0.05 (**), ***P < 0.01 (***) or ****P < 0.001 (****).

Discussion

C. jejuni is the leading cause of foodborne gastroenteritis and, therefore, remains a major problem for the poultry industry (Wassenaar, 2011). Despite this, C. jejuni pathogenesis in poultry is still poorly understood, except that the infection commences in the gastrointestinal tract but can also become extra-intestinal (Chaloner et al., 2014). Results of our previous study indicate that C. jejuni increases epithelial permeability and consequently may support its own dissemination (Awad et al., 2015a). Although not tested in this study, it seems possible that bacterial invasion into polarized epithelial cells depends on the modification of junctional physiology and paracellular passage of the organism (Konkel et al., 2005). Lamb-Rosteski et al. (2008) also demonstrated that the infection with C. jejuni disrupted tight junctional claudin-4 and increased transepithelial permeability; however, whether this promotes translocation of other luminal bacteria in vivo is unknown.

From the present and our previous studies, we hypothesize that the effects of C. jejuni on the intestinal epithelial cells may also promote the translocation of other microorganisms, such as E. coli. Our actual results demonstrate that numbers of E. coli were lower in the jejunum and cecum of infected birds compared to controls. A similar result has recently been shown by Sofka et al. (2015) who found that the enumeration of E. coli was lower in cecal samples positive for C. jejuni, in comparison to Campylobacter-negative birds. Further to this, the present findings revealed that C. jejuni increases the translocation of E. coli to
Figure 2. Changes of short-chain fatty acids concentrations in the digesta (μmol/g) of (A) jejunum and (B) cecum after Campylobacter infection. Data are presented as the mean values and SEM (n = 10). Asterisks mark differences to resp. control with $P < 0.1$ (‘’), $P < 0.05$ (‘’), or $P < 0.001$ (‘’’’).
internal organs, the liver and spleen, of infected birds. This process might be supported via a higher intestinal permeability according to Ferrier et al. (2003) who demonstrated that bacterial translocation correlated with the intestinal permeability (leaky gut). Therefore, our findings are in agreement with Kalischuk et al. (2009, 2010) who found that Campylobacter promotes the translocation of non-invasive bacteria across the intestinal epithelium of mice and humans. Interestingly, in epidemiological studies, it was also reported that an increase of E. coli in whole chickens or neck skin samples taken from broiler carcases throughout processing was associated with an increase of Campylobacter (Habib et al., 2012; Duffy et al., 2014).

The gastrointestinal tract of the animal host is an extremely complex ecosystem in which the mucus layer and the underlying epithelium compose a part of the ecosystem and they are the first line of defence against invading microorganisms. In previous studies, it was found that a Campylobacter infection modulates mucous production, by increasing the viscosity values of the intestinal content (Molnar et al., 2015) and exerts morphological changes in the jejunum of broiler chickens (Humphrey et al., 2014; Awad et al., 2015a). However, most aspects of C. jejuni interaction with the natural ecosystem of the chicken gut are still unknown, especially with regard to the microbial fermentation. Such knowledge could be useful for understanding the cascade of events during C. jejuni colonization in chickens. Therefore, possible alterations in the bacterial metabolites (short-chain fatty acids, SCFAs) in response to Campylobacter infection were investigated.

SCFAs play a major role in the physiology of the intestinal mucosa due to their effect on the expression and activity of nutrient transporters in the intestinal brush border membrane which influences nutrient acquisition (Tappenden et al., 1997). Among the bacterial fermentation end products in the chicken cecum, butyrate is of particular importance because of its nutritional properties for the epithelial cell and pathogen inhibitory effects in the gut (Sun and O’Riordan, 2013). Our results showed that Campylobacter had the ability to reduce butyrate, isolubutyrate, valerate, and isovalerate in the cecum at 7 and 14 dpi, suggesting that Campylobacter infection was associated with changes in bacterial metabolic activity. This suggestion is coherent with results from another study (unpublished data) which we performed, demonstrating that Campylobacter colonization markedly increased the luminal pH of the jejunum and cecum of the infected birds by approximately 0.5 pH units to ~6.7 (jejunum) and ~7.0 (cecum), which is the optimum for Campylobacter growth (Keener et al., 2004).

In addition, results from the actual study indicate that Campylobacter colonization depends more on butyrate and valerate rather than acetate. It remains to be shown whether the altered SCFA concentrations are primarily due to altered production or also due to altered clearance of these SCFAs from the infected birds’ gut. Irrespectively of the latter, however, the altered SCFAs concentrations likely favour the colonization of the gut by this bacterium. This postulation is supported by findings of Masanta et al. (2013), who reported that metabolic end products derived from the intestinal microbiota support C. jejuni to colonize the human gut and to invade epithelial cells, since it was shown that C. jejuni utilizes short-chain fatty acids as a carbon source (Masanta et al., 2013).

In general, changes in the fermentation of end products are most likely due to the activity and density of the resident microbiota. Therefore, the results of the present study substantiate that the Campylobacter infection has a certain influence on the development of the microbial populations and their activity, which needs to be further investigated. Bereswill et al. (2011) demonstrated that a shift of intestinal microbiota was linked with increased susceptibility for C. jejuni infection. Kaakoush et al. (2014) showed that Escherichia coli was a major contributor in chickens colonized with C. jejuni and the SCFAs produced by these bacteria are most likely used as energy sources for C. jejuni and affect its ability for colonizing the chicken gut. Furthermore, alterations in SCFAs concentrations might increase translocation of pathogenic bacteria to extra-intestinal sites by affecting the virulence of bacteria via providing a signal for expression of invasion genes (Lawhon et al., 2002).

In conclusion, Campylobacter can alter gut colonization dynamics and physiologic processes due to the change in the end products metabolised by the gut flora. Furthermore, Campylobacter promotes the translocation of luminal bacteria with possible consequences on animal health. In this context, this work provides new insights into C. jejuni pathogenesis and illustrates the bacterial–host crosstalk during infection in chickens. However, further investigations are needed to resolve the basis of the mucosal response under physiological and pathological conditions as such processes have implications on animal health and welfare.

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