Abstract

Campylobacter enteritis is the most reported zoonotic disease in many developed countries where it imposes a serious health burden. *Campylobacter* transmission to humans occurs primarily through the chicken vector. Chicks are regarded as a natural host for *Campylobacter* species and are ~~mostly~~ colonized with *C. jejuni* in particular. But despite carrying a very high bacterial load in their gastrointestinal tract these birds, in contrast to humans, do not develop pathological signs. It seems that in chickens *C. jejuni* principally harbours in the cecal mucosal crypts, where an inefficient inflammatory response fails to clear the bacterium from the gut. Recent intensive research resulted in an increased insight into the crosstalk between *C. jejuni* and its avian host. This review discusses the chicken intestinal mucosal immune response upon *C. jejuni* entrance, leading to tolerance and persistent cecal colonization. It might in addition provide a solid base for further research regarding this topic aiming to fully understand the host-bacterium dynamics of *C. jejuni* in chicks and to develop effective control measures to clear this zoonotic pathogen from poultry lines.

Keywords: *Campylobacter jejuni*; broiler chicken; immune response; tolerance; persistent colonization

Introduction

From 2005 onwards, *Campylobacter* enteritis has been the most reported zoonotic disease in many developed countries (EFSA, 2011). A~~nd a~~lthough mostly self-limiting, several sequelae might be developed, such as Guillain-Barré syndrome, reactive arthritis, irritable bowel syndrome and inflammatory bowel disease, which can eventually result in mortality (EFSA, 2010). Thus, campylobacteriosis poses a serious health burden in developed countries, where disease in humans is mostly caused by pathogenic *C. jejuni* strains (EFSA, 2011). Chickens are a natural host for *Campylobacter* spp. and are often colonized with *C. jejuni* in particular (EFSA, 2011). This review will therefore mainly focus on the interaction of *C. jejuni* with the chicken host. Despite being colonized in their ceca at a high degree, broiler chickens do not show typical signs of pathology and carry a high *C. jejuni* load until slaughter. As a consequence, slaughter and carcass processing of such animals result~~s~~ in the contamination of their meat products (Rosenquist et al., 2006), which are major sources for transmitting this pathogen to humans (EFSA, 2010). ~~In addition, *C. jejuni* is frequently found in the intestines of broiler roosters and laying hens too (Cox et al., 2009). A decline in human cases of campylobacteriosis is not bound to happen as long as the poor understanding on the host-bacterium interactions of *C. jejuni* in its chicken host hampers.~~ U~~ntil recently~~p to now~~, the knowledge on the chicken immune response in general ~~is~~ has been poor, hampering the development of control strategies to eradicate *C. jejuni* from poultry animals (Hermans et al., 2011a). However, intensive research in the past few years has resulted in an increased insight into

the chicken immune response toward *C. jejuni* ~~entrance~~colonization. This review discusses the ~~dual~~ interaction between *C. jejuni* and its chicken host, focussing on immune responses, leading to persistent, high-level cecal colonization. At the end of this review the mechanisms that are potentially responsible for the redirection of this response toward tolerance, and thus for the different disease outcome compared to humans, are handled.

Colonization pattern and antigenic variation of *Campylobacter jejuni* in chicks

Despite some reports of *Campylobacter*-induced diarrhea, systemic invasion, growth retardation and jejunal villus atrophy (Ruiz-Palacios et al., 1982; Sanyal et al., 1984; Sang et al., 1989; Lam et al., 1992; Lamb-Rosteski et al., 2008) it is generally accepted that *C. jejuni* colonizes the avian gut as a commensal. Colonization of chickens with *C. jejuni* does not cause clinical illness nor changes in cecal mucosa morphology even though large numbers of the bacterium reside ~~in their cecat~~here (generally around 10^6 to 10^8 cfu/g), the predominant site for colonization (Beery et al., 1988; Van Deun et al., 2008; Meade et al., 2009b). Commensal bacteria in general do not colonize outside the gastrointestinal (GI) tract, but strangely enough *C. jejuni* can readily be found in various extraintestinal organs of broilers too. Up to seven days after oral and cloacal inoculation, the bacterium was found in the thymus, spleen, liver/gallbladder and bursa of Fabricius (Cox et al., 2005; Van Deun et al., 2008; Meade et al., 2009b). In a study with two-week-old chicks that were inoculated with *C. jejuni* at day-of

hatch, high bacterial numbers (> 5 log CFU/g) were isolated from spleen and liver of most of the birds (Lamb-Rosteski et al., 2008). In addition, *C. jejuni* was isolated from the reproductive tract and ovarian follicles of laying hens (Cox et al., 2009). The dissemination of *C. jejuni* to other organs seems to be correlated with the invasive potential in primary cecal epithelial cells of chicks (Van Deun et al., 2008), suggesting that *C. jejuni* translocates the epithelial barrier transcellularly (through the chicken crypt epithelium) rather than paracellularly (between cells).

Upon ingestion, *C. jejuni* reaches the cecum and multiplies, resulting in an established colonizing population within 24 h after infection (Coward et al., 2008; Smith et al., 2008). Most broiler flocks become colonized only at an age of two to four weeks after which the infection rapidly spreads to almost all birds (>95%), which remain colonized until slaughter (Jacobs-Reitsma et al., 1995; Stern et al., 2001; Stern, 2008; van Gerwe et al., 2009). Although not all birds in a flock were colonized, it was demonstrated that *C. jejuni* can be isolated from laying hens until an age of 42 weeks (Lindblom et al., 1986) and probably longer, since experimental periods exceeding one year are not documented. This implies that *C. jejuni* can evade the chicken host immune system. However, in a study by Cawthraw and Newell (2010) colonization of breeder birds decreased over time, indicating ~~and resist elimination by~~some mucosal clearance. In addition, with older birds it cannot be ruled out that replacement of one strain by an immunologically distinct strain (strain succession) occured, disguising mucosal clearance of the former *C. jejuni* strain.

Campylobacter-positive flocks are often colonized with more than one sero- or genotype at the same time (referred to as co-colonization), which may be explained by recurring environmental exposure to the bacterium but also by genetic changes within the *C. jejuni* population (van de Giessen et al., 1992; Jacobs-Reitsma et al., 1995). The dominating strains are replaced throughout the colonization period, probably due to strain-specific immune responses, and it seems that this colonization pattern is mainly determined by the chicken host and not by the host microbiota (Skanseng et al., 2007; Ridley et al., 2008). Indeed, different breeds of chicken may differ in their susceptibility to colonization by *C. jejuni* (Stern et al., 1990; Boyd et al., 2005). It has been suggested that a paternal effect might be an important genetic factor influencing resistance to *C. jejuni* colonization in broilers (Li et al., 2008). However, there are also other lines of evidence suggesting that external factors are responsible for the *Campylobacter* colonization pattern in broilers. It has been found in artificially inoculated birds that different *C. jejuni* genotypes may compete for colonization leading to a *C. jejuni* succession in broilers (Konkel et al., 2007).

C. jejuni isolates often show increased colonization potential after passage through the chicken gut (Ringoir & Korolik, 2003). Chicken intestinal colonization may favour genetic recombination in *C. jejuni*, resulting in different *flaA* types, ribo- and PFGE patterns (Hanninen et al., 1999; Van Deun et al., 2007; Hanel et al., 2009). Interstrain genetic exchange and intragenomic alterations were shown to occur *in vivo*, even in the absence of selective pressure (de Boer et al., 2002). It has been demonstrated that bacteriophage genes are known to be present

in the genome of *C. jejuni* and that phages can alter PFGE patterns of this bacterium (Barton et al., 2007; Clark & Ng, 2008). Both phage-dependent and -independent rearrangements of the genome result in an enormous antigenic variation among *C. jejuni* isolates with the former resulting in phage-resistant *C. jejuni* types (Scott et al., 2007a, 2007b). Besides protection against phage predation, this generation of antigenic diversity may also play an important role in immune evasion and thus in chicken gut colonization. However, *C. jejuni* strains that underwent rearrangements leading to phage-resistance were demonstrated to be inefficient colonizers of the chick intestine (Scott et al., 2007b). There is still some controversy regarding the genomic instability of *C. jejuni* since Nielsen et al. (2001) concluded that many strains were genetically stable as tested by riboprinting, PFGE, RAPD and Penner heat-stable serotyping after *in vitro* and *in vivo* (through mice) passage. Moreover, Manning et al. (2001) concluded that this stability could be maintained despite exposure to various environmental conditions over long time periods and covering large distances. Also, it has been suggested that subtype pattern variations in *C. jejuni* leading to phenotypic changes, occur only occasionally during *in vivo* passage (Konkel et al., 2007). On the other hand, Ridley et al. (2008) observed that, although stable during single cecal colonization of one individual strain, the *C. jejuni* genome can undergo changes upon competitive stress (i.e. during co-colonization) in the avian gut, leading to PFGE type variants with different colonization capacities from a single parent clone. This genetic and phenotypic diversity might play a role in the improved fitness of certain *C. jejuni* strains to survive and colonize another host.

Crosstalk between *C. jejuni* and the chicken gut mucosa

Colonization mechanism

Although *C. jejuni* is likely to encounter environmental stressors compromising optimal growth in its chicken host (Murphy et al., 2006), the bacterium persistently colonizes the chicken gut. This indicates that the bacterium harbours regulatory systems conferring protection toward a hostile environment inside its host. Although it is clear that successful colonization of the chicken GI tract is a multifactorial process (Newell, 2002), the mechanism by which *C. jejuni* is able to persistently evade the chicken immune response is poorly understood.

Upon entering the chicken GI tract, *C. jejuni* moves toward the intestinal epithelial border, probably mediated by chemotaxis. *C. jejuni* is attracted by intestinal mucins, as well as several amino acids, carbohydrates and salts of organic acids, while the chemoattractive properties of L-fucose are controversial (Vegge et al., 2009). *C. jejuni* responds to these chemicals via methyl-accepting chemotaxis proteins (MCP) (Vegge et al., 2009), of which the most important are the determinant of colonization proteinB (DocB) and the chemoreceptor transducer-like protein1 (Tlp1), while the chemotaxis regulatory proteinY (CheY) shuttles between these MCP and the flaggelar motor (Hendrixson & DiRita, 2004; Hartley-Tassell et al., 2010). The putative adaptation system CheBR is believed to be involved in the response of *C. jejuni* to these environmental signals by modifying its chemoreceptors (including Tlp1) (Kanungpean et al., 2011). DocB and Tlp1 truncation, however, does not alter the chemotactic behaviour of *C.*

jejuni in vitro, ~~indicating that they either serve partly redundant chemotactic functions or a different function. Indeed, these MCP proteins were shown to~~but rather reduces its invasive potential in chicken embryo intestinal cells (Vegge et al., 2009). In any case, there is no doubt that DocB and Tlp1 are indispensable for *C. jejuni* to colonize chicks~~ the *in vivo* function of these proteins, as well as chemotaxis regulation *in vivo* in general, remain somewhat obscure~~. For moving toward the most favourable conditions for growth *C. jejuni* needs intact flagella and it seems that especially *flaA*, *flgK*, *cj1324* and the motility accessory factor *maf5* gene are crucial for colonizing the chicken gut (Hermans et al. 2011b).

The host intestinal mucus layer that lines the epithelial cells prevents most commensal bacteria to make direct contact with the epithelial surface by constituting a viscous physical barrier and by harbouring secretory IgA and antimicrobial peptides (Ivanov & Littman, 2011). And although increased viscosity has been associated with down-regulation of *flaA* promoter activity (Allen et al., 2001), the modified flagellum of *C. jejuni* allows the bacterium to penetrate the viscous mucus layer (Guerry, 2007) and ~~to reach and~~from making directe contact with the intestinal epithelial cells. Although *C. jejuni* is not found to be attached to chicken cecal crypt microvilli *in vivo* (Beery et al., 1988) the bacterium has been observed intracellularly in intestinal epithelial cells of three-day-old experimentally inoculated chickens and in chicken primary cecal epithelial crypt cells *in vitro* (Van Deun et al., 2008). Moreover, several adhesins of *C. jejuni* have been implicated to be important for chick colonization. Therefore, upon entering the chicken gut it is believed that *C. jejuni* adheres to the

epithelial cells, mediated by intact flagella and surface-exposed proteins. In particular CadF (*Campylobacter* adhesion to fibronectin) ~~(CadF)~~ and FlpA (fibronectin-like protein A ~~(FlpA~~) were identified as important adhesins for colonization, while the potential contribution of *Campylobacter* adhesion protein A (CapA) is less clear ~~*Campylobacter* adhesion protein A (CapA) has been implicated as a putative adhesion (Ashgar et al., 2007). In contrast, in another study no reduced colonization in chicks was observed for a *capA* mutant, although *C. jejuni* adherence to chicken LMH cells was attenuated (Flanagan et al., 2009). This study also revealed that *capA* is not conserved among *C. jejuni* isolates, suggesting only a limited role for CapA during chicken colonization~~.(Hermans et al. 2011b) Also several surface-accessible carbohydrate structures of *C. jejuni*, such as lipooligosaccharide (LOS) and an intact capsule, are involved in adhesion with in particular the capsular polysaccharide transporter gene *kpsM* and the *N*-linked general protein glycosylation pathway gene *pglH* being important for colonization of the chicken intestinal tract (Karlyshev et al., 2004; Hermans et al., 2011b).

Adhesion of *C. jejuni* to gut epithelial cells ~~is probably~~may be followed by marginal invasion in these cells. Upon exposure to chicken mucus, the flagellar apparatus increases the secretion of *Campylobacter* invasion antigens (Cia), important for *in vitro* cell invasion and chick colonization (Ziprin et al., 2001; Konkel et al., 2004; Biswas et al., 2007). Also *C. jejuni* LOS is important for epithelial cell invasion as well as for immune evasion in humans and sialylation of the LOS outer core further enhances these traits (Louwen et al., 2008; Habib et al.,

2009). *C. jejuni* is not able to suvive for long periods in primary chicken cecal epithelial cells, nor is it able to multiply in cultured human intestinal epithelial cells. Therefore, intracellular replication in these cells is probably not important for persistant *in vivo* colonization. Rather, invasion of cecal crypt epithelial cells would be followed by evading these cells allowing *C. jejuni* to replicate in the mucus, which seems to provide all necessary nutrients for optimal growth, and re-invasion to escape mucosal clearance (Van Deun et al., 2008). Strangely, in contrast to Caco-2 invasion, the invasion capacity of *C. jejuni* in primary chicken cecal epithelial cells *in vitro* is not correlated with *in vivo* gut colonization, but is with systemic dissemination (Van Deun et al., 2008). Therefore, the genuine contribution of epithelial cell invasion during cecal colonization of chicks with *C. jejuni* is not clearly definable and can only be speculated on.

~~Next~~ In addition to these three key events (chemotaxis, adhesion and possibly invasion), ~~also~~ a plethora of additional mechanisms, including several stress responses, multidrug and bile resistance regulation, iron regulation and energy metabolism are definitely important for initial and persistent high-level colonization of the avian GI tract with *C. jejuni* (Hermans et al. 2011b).

Chicken intestinal immune response ~~upon~~ to *C. jejuni* ~~entrance~~colonization

Protection of young chicks against C. jejuni colonization

Day-of-hatch chicks have no established gut flora and possess an immature mucosal immune system. In the cecum, it is only after four to seven days post-hatch that an increase in cecal pro-inflammatory chemo- (such as interleukin-8

(IL-8)) and cytokine expression and heterophil numbers can be observed, upon exposure to feed and microflora (Bar-Shira & Friedman, 2006). Hatchlings are also unprotected by adaptive immunity, which only starts to develop after a few days of life (Friedman et al., 2003). Nevertheless, colonization of chickens with *C. jejuni* during this critical period seems not to occur. Instead, maternally-derived antibodies generated against flagellin proteins (such as FlaA), adhesins (such as CadF) and other *C. jejuni* surface components are important in protecting young chickens from *C. jejuni* colonization during the first two weeks, the so called lag-phase (Sahin et al., 2001, 2003; Shoaf-Sweeney et al., 2008; Zeng et al., 2009). Killing of *C. jejuni* by maternal antibodies happens in a complement-mediated, strain-specific way (Young et al., 2007). These antibodies confer enhanced protection against challenge with a homologous strain compared to a heterologous strain, probably because they retard motility of a homologous, but not that of a heterologous strain, as shown *in vitro* (Sahin et al., 2003). After the lag-phase, chickens show an increased susceptibility to colonization with *C. jejuni* which coincides with a loss of maternally derived, circulating anti-*Campylobacter* IgY antibodies, suggesting that adaptive immunity is not critical in protecting broilers from colonization (Cawthraw et al., 2010). Interestingly, day-of-hatch broilers have been shown to be very susceptible to *C. jejuni* colonization, which again diminished over the first few days of life (Cawthraw et al., 2010; Conlan et al., 2011), while transmission of *C. jejuni* between co-housed birds is lower in day-old chicks compared to two-week-old birds (Conlan et al., 2011). This indicates

that a lack of exposure of broiler flocks to *C. jejuni* and/or reduced transmission during the early stages of rearing may also contribute to the observed lag-phase.

Developing chicken embryos have increased expression levels of several avian *β*-defensins, a group of antimicrobial peptides important in innate and adaptive immune responses that might contribute to the observed protection toward *C. jejuni* infection *in ovo* and post-hatch (Meade et al., 2009a). For the *β*-defensin gallinacin-6, for instance, *in vitro* antibacterial activity against *C. jejuni* has been demonstrated (van Dijk et al., 2007).

Innate immune response

The chicken intestinal innate immune system ~~is built up by~~comprises several tissues, cells (such as epithelial cells, monocytes/macrophages, dendritic cells, natural killer cells and neutrophils) and germline-encoded molecules (such as chemo- and cytokines, antimicrobial peptides and nitric oxide) that can limit both commensal and pathogenic invading bacteria (Brisbin et al., 2008). Some *in vitro* studies with macrophages and epithelial cells, both primary and cultured, contributed to the insight into the chicken immune response toward *C. jejuni* infection. *C. jejuni* has been shown to be adhesive to, invasive in and to stimulate inflammatory responses from these cells (Smith et al., 2005; Byrne et al., 2007; Larson et al., 2008; Van Deun et al., 2008). Evidence of both *in vitro* uptake of *C. jejuni* by chicken peritoneal macrophages (Myszewski & Stern, 1991) and *in vivo* presence of *C. jejuni* within chicken epithelial cells and macrophages (Ruiz-Palacios et al., 1991) exists.

A crucial step in the host innate immune response to bacterial entrance in the GI tract is the activation of Toll-like receptors (TLRs), expressed on a variety of cells of the GI mucosa including macrophages and epithelial cells, the latter forming the first borderline defence against invading pathogens (He et al., 2006; Linde et al., 2008). TLRs are recognized by specific bacterial ligands and, once activated, promote the expression of effector molecules such as antimicrobial peptides, NO and inflammatory cytokines. Although knowledge on avian TLR biology is only starting to unravel, very recently several chicken TLRs have been implicated to play a role in *C. jejuni* recognition. The chicken TLR4/myeloid differentiation protein-2 (chTLR4/chMD-2) complex and cell-surface expressed chTLR2 recognize *Campylobacter* LOS and lipopeptides, respectively. Both receptors are potently activated by lysed *Campylobacter* bacteria. However, loss of bacterial cell wall integrity does not seem to play a critical role in TLR activation, because also live *Campylobacter* bacteria are able to elicit a marked inflammatory response in chickens (de Zoete et al., 2010). TLR5 specifically recognizes conserved regions of bacterial flagellins, thereby preventing intestinal pathology. *C. jejuni*, however, lacks these TLR5-recognition sites and is therefore unable to activate chTLR5, indicating that TLR5 signaling does not play a critical role in the chick immune response against *C. jejuni* (Guerry, 2007; de Zoete et al., 2010). Finally, TLR21, which is unique to avian, amphibian and fish species, enables recognition of unmethylated single stranded microbial 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA motifs with a broad ligand specificity. *C. jejuni* CpG DNA is internalized through endocytosis and most

likely interacts with chTLR21 intracellularly, similar to the interaction of CpG DNA with the functional homologue (TLR9) in mammals (de Zoete et al., 2010, Keestra et al., 2010).

Activation of chTLR2, chTLR4 and chTLR21 results in an innate immune response through myeloid differentiation primary response gene 88 (MyD88)-dependent activation of nuclear transcription factor kappaB (NF-κB) and subsequent production of inflammatory cytokines and chemokines (Brownlie et al., 2009; de Zoete et al., 2010; Keestra et al., 2010). Additionally, chTLR4 and chTLR21 ligands can induce the production of inducible nitric oxide synthase-mediated NO from chicken monocytes (He et al., 2006). In mammals, TLR-signaling also involves a TLR4-mediated MyD88-independent pathway associated with the induction of late phase NF-κB and interferon (IFN)-inducible genes, such as IFN-β, involved in natural killer cell activiation, and maturation of dendritic cells (Yamamoto et al., 2004). Chickens, however, lack this pathway and therefore have an aberrant response to *C. jejuni* LOS compared to mammalian species, rendering them much more resistant to the toxic effects of these TLR4 agonists. Although the TLR4-mediated MyD88-dependent pathway, leading to early phase activation of NF-κB, is intact, this explains in part the absence of pathological signs in chicks in response to infection with *C. jejuni*, despite cell adhesion and invasion (Keestra & van Putten, 2008; Shaughnessy et al., 2009; de Zoete et al., 2010).

Upon *Campylobacter* infection, primary chick kidney cells and the avian macrophage cell line HD11 express NO and pro-inflammatory cyto- (IL-6 and IL-

1β) and chemokines (chIL-8) (Larson et al., 2008). Production of NO by activated macrophages is important for their bactericidal activity (Linde et al., 2008). IL-1β and IL-6 are both major mediators of the innate immune system, while IL-6 is also involved in the immunological switch from innate to adaptive immunity (Smith et al., 2005). IL-1β is primarily produced by monocytes/macrophages and is involved in the inflammatory response of chickens against microbial products (such as lipopolysaccharide (LPS)) by instructing epithelial cells and macrophages to produce chemokines (Bar-Shira & Friedman, 2006). The chicken orthologue of mammalian IL-8 (CXCLi1 and CXCLi2, but here referred to as chIL-8) (Kaiser et al., 1999; Smith et al., 2005) attracts heterophils and, unlike its mammalian counterpart, also monocytes to the site of infection (Martins-Green, 2001). It has been demonstrated that the *N*-terminus of chIL-8, where the chemotactic activity resides, is structurally homologous to that of monocyte chemotactic protein-1 (Borrmann et al., 2007). This human chemokine is chemotactic for monocytes, probably explaining the chemotactic movement of monocytes toward chIL-8. A marked chIL-8 response is induced in chicken LMH and primary intestinal cells upon inoculation with *C. jejuni* (Brisbin et al., 2008; Li et al., 20010). Finally, also in chicken embryo intestinal cells *C. jejuni* is capable of inducing a pro-inflammatory response (Smith et al., 2008; Li et al., 2010).

Despite the lack of association of *C. jejuni* with chicken crypt epithelium *in vivo*, some recent reports demonstrate the initiation of a mild inflammatory response in chickens upon exposure to the bacterium. *C. jejuni* colonization in chickens is

accompanied by infiltration of proinflammatory cells in mucosal tissues, although overt signs of cell invasion or pathology were not found (Larson et al., 2008; Smith et al., 2008). Upon inoculation of four-week-old broilers, an early increase (six h post- inoculation (pi)) in circulating monocytes/macrophages was observed and increased numbers were maintained after 48 h (Meade et al., 2009b). Strikingly, heterophil numbers remained unaltered during this time course. Absence of a heterophil infiltrate was also observed in cecal mucosal tissues of three-week-old hens 24 h after direct~~ly~~ injection of their cecum~~ed~~ with *C. jejuni* (Van Deun et al., 2008). In contrast, another study (Smith et al., 2008) showed a minor, although significant induction of heterophil infiltration in cecal tissues one day and four days after inoculating two-week-old broiler chicks, as well as in the ileum at four days post-inoculation. It cannot be ruled out that also in the studies by Meade et al. (2009b) and Van Deun et al. (2008) a heterophil influx could have been observed after four days, but the discrepancy in heterophil influx after one day between these studies is not clear. Possibly, the differenct *C. jejuni* strains used in these studies may have accounted for this. But more likely, differences in chicken lines and bird age were responsible in the differential host response because in the study by Smith et al. (2008) an out-bred flock was used and the heterophil influx observed in two-week-old birds was absent in day-of-hatch chicks. In one-day-old birds, however, this induction was not observed. Expression of both TLR4 and TLR21, but not TLR2, is readily increased (six h pi) in cecal tissues in response to *C. jejuni* inoculation (Meade et al., 2009b; Shaughnessy et al., 2009). In two- and four-week-old broiler chicks this is

accompanied, however, by only a limited cytokine gene expression except for a marked increase in *chIL-8* expression ~~already~~ after 6-12 h pi which is maintained over 48 h after inoculation (Shaughnessy et al., 2009) and longer (Smith et al., 2008). *IL-1β* expression levels are moderately increased after 20-24 h and decrease afterwards, while increased *IL-6* expression is evident only after 48 h at the earliest (Keestra & van Putten, 2008; Smith et al., 2008). A similar response can be observed in ileal tissues although a marked induction of *IL-6* expression levels was already evident in these tissues at six h pi after which they started to drop again. In one-day-old chicks, these reponses are less pronounced or absent although also in these animals *IL-8* expression in cecal tissues is induced. Overall, induction of cytokines is most evident within 24 h after inoculation after which the expression levels drop again. Because the intestinal bacterial load in these *Campylobacter*-colonized chicks did not lower during the examined time-course, there clearly exist some mechanisms that are responsible for controlling this pro-inflammatory response (Smith et al., 2008). Expression levels of anti-inflammatory *IL-10*, *IL-13* and transforming-growth factor *β*4 (*TGF-β4*) were not detected in cecum, ileum and spleen, and the signals modulating the pro-inflammatory response, resulting in sustained and unaffected *C. jejuni* colonization, are yet unknown (Smith et al., 2008; Shaughnessy et al., 2009). *C. jejuni* colonization in chicks significantly reduces expression levels of several antimicrobial peptide genes (Meade et al., 2009a). This downregulation might represent one mechanism whereby *C. jejuni* modulates the immune response, limiting the efficacy of these antimicrobial factors and enabling itself to

persistently colonize its host at high levels. As stated above, gallinacin-6 has a bactericidal effect on *C. jejuni* (van Dijk et al., 2007). Based on mRNA levels, expression of this defensin is low in the avian intestinal tract, and no detailed studies have been done upon the time of writing this review that indicate an inducible upregulation of gallinacin-6 after exposure to *C. jejuni*. In a recent study by Shaughnessy et al. (2011) 270 genes were found to be significantly ($P < 0.01$) differentially expressed after 20 h in four-week-old chicks colonized with *C. jejuni* compared to *C. jejuni* free chicks. These genes corresponded to the activation of several biological processe, including immue responses. Although differences in expression were only marginal, this response was hypothesized to point toward an innate T-cell response in the ceca of chickens 20 h after inoculation with *C. jejuni* (Shaughnessy et al., 2011).

Adaptive immune response

The type of immune response generated against *C. jejuni* depends on the cytokine microenvironment induced by the chick innate defence cells. This in turn is determined by the interaction of TLRs and other pathogen recognition receptors expressed on these cells with their respective ligands. In chickens, not all of these receptors and cytokines are fully identified yet, making the switch from innate to adaptive immunity in this species not completely understood (Brisbin et al., 2008).

In chickens, intestinal antigens are capable of entering the bursa of Fabricius, the site of primary B cell development (Brisbin et al., 2008). Chickens have an

incomplete antibody response toward T-cell independent type 2 antigens which activate B cells independently of T cells (Jeurissen et al., 1998). Because these antigens are usually of polysaccharide nature, an insufficient humoral response toward certain surface-accessible carbohydrate structures (SACS) of *C. jejuni* might contribute to the inability of the chicken immune system to clear this microorganism, despite the antigenic potential of *C. jejuni* LOS and its capsule (Oza et al., 2002) and the marked immunogenicity of *C. jejuni* flagellin (Widders et al., 1998). Moreover, an outer membrane protein extract of *C. jejuni* has been shown to cause apoptosis of chicken blood and spleen lymphocytes, probably promoting immune evasion of *C. jejuni* in the chick (Zhu et al., 1999). An antibody response to *C. jejuni* might, however, contribute to protection against intestinal colonization of chickens, which show a significant increase in specific mucosal and circulating IgG (IgY) and IgA and circulating IgM antibody titres when colonized with *Campylobacter* (Cawthraw et al., 1994; Widders et al., 1998). In these studies flagellin was shown to be the immunodominant antigen, which is rather peculiar due to the lack of functional TLR5-recognition sites in *C. jejuni* flagellin, permitting TLR5 evasion (Guerry, 2001; de Zoete et al., 2010). Nevertheless, vaccinating chicks with a hybrid protein based on *C. jejuni* FlaA induced a specific response against this protein and reduced colonization in these birds (Khoury and Meinersmann, 1995). An antibody response specific for native flagellin was also induced in the serum of chickens immunized with purified *C. jejuni* flagellin. Serum and GI secretion antibodies specific for *C. jejuni* whole cells were, however, only induced ~~But only~~ when ~~the~~ this protein was

complemented with killed *C. jejuni* whole cells. ~~which moreover~~ resulting~~ed~~ in reduced cecal *C. jejuni* counts in these birds (Widders et al., 1998). This might indicate that the epitopes of *C. jejuni* flagella are not accessible for these antibodies in intact bacteria and that possibly other antigens, not detected in this study, were responsible for the induction of anti-*C. jejuni* antibodies reducing the cecal bacterial load. Recent studies gave more insight into this enigma and identified additional immunogens of *C. jejuni* promoting the humoral immune respons in chicks. Amongst others the *C. jejuni* ferric enterobactin receptor CfrA (involved in iron regulation), the outer membrane chanel CmeC (involved in multidrug resistance), Cj0091 (belonging to a lipoprotein-encoding operon), the lipoprotein CjaA and CjaC (mediating amino acid transport), CadF and LOS were shown to be immunogenic and expressed during *in vivo* colonization (Shoaf-Sweeney et al., 2008; Zeng et al., 2009; Oakland et al., 2011). Both the sera of young chicks free of *C. jejuni* and older birds colonized with the bacterium were reactive against recombinant CfrA, indicating that they are not only passed from the mature hen to the hatchling but are also induced during colonization of broilers after the lag-phase (Zeng et al., 2009). It was speculated that antibodies directed to CfrA hinder the interaction of FeEnt with its receptor. Proper functioning of CfrA is crucial for *C. jejuni* colonization in chicks, indicating that CfrA antibodies are potentially protective. Also *C. jejuni* CjaA-based vaccines were shown to induce specific serum IgY and mucosal IgA antibody responses against CjaA and reduced cecal colonization of vaccinated chickens (Buckley et al., 2010).

Intestinal epithelial cells might contribute to a mucosal IgA response by the GALT, located beneath the epithelial cell border in the lamina propria, in a T-cell dependent manner by producing IL-6 after contact with *C. jejuni* (Faragasan, 2008). Secretory IgA is the major immunoglobulin isotype in mucosal secretions and generally responsible for preventing sub-epithelial translocation of commensal bacteria by preventing their adhesion to epithelial cells or returning bacteria that already reached the basolateral site, without eliciting an inflammatory response (Brisbin et al., 2008). Moreover, by its resistance to normal intestinal proteases, through dimerization on the surface of mucosal epithelial cells, IgA is ideally suited for host defences at the mucosal surface of the GI tract (Phalipon et al., 2002). IgA might thus play an important role in limiting the mucosal immune response to *C. jejuni* in chickens and redirecting it toward tolerance.

Most *C. jejuni* strains possess genes encoding a cell death-promoting cytolethal distending toxin (CDT) of which the expression is induced in both the avian and human gut (Abuoun et al., 2005). During human infection with *C. jejuni*, neutralizing antibodies against CDT are induced, but not during colonization in chickens and it seems that production of this toxin in general is not important for chick colonization as opposed to its suspected role during pathogenesis in humans (Abuoun et al., 2005; Biswas et al., 2006).

As mentioned above, genetically distinct chicken lines may differ in their susceptibility toward cecal *C. jejuni* colonization (Stern et al., 1990). Further research in this area revealed insulin receptor signaling and metabolism process

pathways to be key players of this differential response (Li et al., 2010). In a more resistant line, lymphocyte activation, lymphoid organ development functions and circadian rhythm were important in the cecal host defence upon *C. jejuni* inoculation. In a more susceptible line, cell differentiation, communication and signaling pathways were important during host defence, with a marked upregulation in lipid, glucose and amino acid metabolism.

Chicken systemic immune response to *C. jejuni*

The frequently observed systemic colonization of *C. jejuni* in chicks indicates that the bacterium, despite the induction of secretory IgA by the GALT, is capable of breaching the gut epithelial barrier. As in the GI tract this happens without developing pathology or inducing excessive inflammation, although chicks can mount an adaptive T cell response to *C. jejuni* when it reaches and colonizes the liver (Jennings et al., 2011). In colonized flocks, almost all birds carry *C. jejuni* in their ceca but significantly less birds harbour the bacteria in their liver tissues (Jennings et al., 2011). Whether host-specific differences decide over *C. jejuni* dissemination, or a T cell response is responsible for the eradication of *C. jejuni* from the host liver in some animals, is not known. In any case, *C. jejuni*-specific antibody responses are apparently not capable of clearing the bacterium from the chicken gut, but nevertheless do indicate that there indeed must have been a preceding close interaction between *C. jejuni* and the host epithelial cells.

The two chicken lines used in the study of Li et al. (2010) also differed in their systemic response to *C. jejuni* (Li et al., 2011). In the spleen, a secondary

lymphoid organ of the avian immune system important for lymphocyte activation, proliferation and differentiation, the response to *C. jejuni* in the more resistant line was characterized, as in the cecum, by lymphocyte activation and differentiation. In addition, splenic host genes for humoral responses and Ig heavy and light chain were upregulated. These responses initiate adaptive immune responses to *C. jejuni* and are probably responsible for an increased genetic resistance to systemic *C. jejuni* colonization. In the susceptible line, genes for regulation of erythrocyte differentiation, hemopoiesis and RNA biosynthesis processes were downregulated. This study also revealed distinct innate defense mechanisms against *C. jejuni* by the two chicken lines. Apoptosis and cytochrome *c* release from mitochondria was associated with increased resistance against *C. jejuni* colonization. Probably, these events induce increased apoptosis of infected host cells, thereby destroying the habitat of the bacteria and contributing to the increased resistance to splenic colonization with *C. jejuni*.

Interaction with the host microbiota

Little is known currently about the effect of the natural avian gut microbiota on the level of *C. jejuni* colonization. In general, host microbiota imposes a colonization barrier for intruding pathogens by competing for nutrients (such as carbon) and host receptors. Their composition, however, can alter the outcome of invading enteric bacteria (by e.g. altering the virulence properties of these bacteria), resulting in either clearance or colonization (Keeney & Finlay, 2011). And although it has been suggested that the colonization pattern of *C. jejuni* in

chicks is mainly determined by the chicken host but not by the host microbiota (Ridley et al., 2008), also the composition of the latter might contribute to the observed colonization pattern. Changes in *C. jejuni* loads in the commercial turkey intestine seemed to correlate to, but are not dependent on, two acute transitions in the cecal microbiota composition during the turkey development phases (Scupham, 2009). With an approach called antibiotic dissection, day-old turkey poults were inoculated with cecal contents of *Campylobacter*-free adult turkeys after which the microbial communities in these poults were modified by different antibiotic treatments. Molecular examination of the constituents of these communities detected that a subtype I of *Megamonas hypermegale* correlated with decreased colonization ability of *C. jejuni*, while a virginiamycin-derived cecal microbiota seemed to be correlated with enchanced colonization ability (Scupham et al., 2010). These results indicate that *C. jejuni* may respond to the presence of specific subsets of the avian gut microbiota. It has, however, to be examined if the effect of these gut microbiota alterations on *C. jejuni* in turkeys also applies to chicks.

Hypothetical mechanism of the interaction between *C. jejuni* and the chicken gut mucosa

The interaction of *C. jejuni* with its avian host is very complex, evidenced by the extensive interplay between several key mediators important in successful and persistent colonization in the chicken GI tract. In chicks, this dual interaction is clearly influenced by both the *C. jejuni* strain and the chicken line involved. The

information reviewed above suggests that, despite the lack of a developed pathology, a pro-inflammatory response is developed in the chicken intestinal mucosa during asymptomatic colonization with *C. jejuni*. Upon *Campylobacter* entrance in the avian GI tract, an early induced production of chIL-8 by intestinal epithelial cells is observed, followed by macrophage recruitment and production of proinflammatory cytokines. This is, however, not accompanied by the recruitment of heterophils (the avian equivalent of mammalian neutrophils) to the site of infection. In a later stage, a specific mucosal IgA response is mounted against *C. jejuni*, but this induction is not capable of clearing the bacterium from the gut. This humoral response is moreover not ~~cap~~able to prevent *C. jejuni* from~~to~~ further interacting with and ~~to~~ translocating~~e~~ across the gut epithelium and to disseminate systemically. Also the specific T cell response that is triggered upon *C. jejuni* entrance in the extra-intestinal organs does ~~neither~~ not result in clearance from these tissues, ~~n~~or pathology. ~~Because~~ *C. jejuni* ~~colonizes the chicken gut persistently,~~ ~~it~~ is thus ~~must be~~ capable of ~~somehow~~ evading ~~this~~ the inefficient host immune response and~~.~~ ~~But also~~ ~~t~~he chicken host might be involved in maintaining homeostasis during persistent colonization (see further). In **Figure 1**, a schematic overview is given of a simplified hypothetical mechanism involved in the interaction of *C. jejuni* with the chicken gut, after lag-phase, leading to successful and persistent colonization of the GI tract, without developing pathology.

Commensal *C. jejuni* colonization in chicks: immunological tolerance?

In mammals commensal infections are characterized by the absence of a neutrophil infiltrate or a classical inflammation as seen during pathogenic infection (MacPherson & Uhr, 2004), indicating that the interaction between *C. jejuni* and its chicken host is indeed of commensal nature. Intestinal homeostasis during commensal colonization requires that a proinflammatory response is rapidly controlled. In mammals not much is known about the host regulatory mechanisms that contribute to tolerance without reducing bacterial numbers, but, restricting the bacteria to the lumen (so they cannot reach the epithelial cells and the immune system) and inducing an anti-inflammatory response are believed to induce a state of "immunological ignorance" (Ivanov & Littman, 2011). Due to a lack of knowledge about the interaction between *C. jejuni* and the chicken immune system it remains unclear how homeostasis is maintained in chickens colonized with *C. jejuni*. An apparent induction of a mild intestinal pro-inflammatory response, the inability to demonstrate upregulation of anti-inflammatory cytokines, occasional invasion of cecal crypt epithelial cells and regular dissemination to extra-intestinal organs upon *C. jejuni* colonization of the chicken host, suggests that their interaction is not a tale of ignorance but rather a cohort of active processes, exerted by the two partners, resulting in "immunological tolerance". *C. jejuni* itself might escape or alter the inflammatory response by, for instance, down-regulating antimicrobial peptide gene expression in the chicken gut, but other potential mechanism(s) or bacterial factor(s) of *C. jejuni* involved in immune evasion are currently not known. Alternatively, ~~also~~

the chicken host might support tolerance to maintain homeostasis during persistent, asymptomatic colonization (Pédron & Sansonetti, 2004).

First of all, the differential composition of the chicken intestinal mucus layer, compared to its human counterpart, probably plays an important role in promoting homeostasis during *C. jejuni* colonization. Chicken intestinal mucins have been shown to reduce the adhesive and especially the invasive capacity of *C. jejuni* in human primary and cultured intestinal epithelial cells (Byrne et al., 2007; Alemka et al., 2010). In contrast, human-derived mucus promotes adhesion and entrance (Byrne et al., 2007). Moreover, MUC2, the most abundantly secreted mucin in the human intestine, is a major chemoattractant for *C. jejuni* and induces the expression of several colonization- and virulence-associated genes (Tu et al., 2008). To date, no such properties have been assigned to chicken mucins. Host intestinal mucins can be either secreted or expressed at the apical surface of the (cecal) mucosal epithelial cells and are readily found to be coated with fucosylated glycans in terminal positions (Stahl et al., 2011). Although the chemotactic properties of L-fucose were not validated by Vegge et al. (2009), it is believed that *C. jejuni* is attracted to, and binds with both mucin and L-fucose. Presence of the latter at certain concentrations might moreover increase *C. jejuni* *flaA* promoter activity (Allen et al., 2001). Therefore, fucosylated glycans may function as adherence factors for *C. jejuni*. In addition, although it was believed until now that *C. jejuni* is an asaccharolytic organism, very recent evidence indicates that some strains are able to use L-fucose as a substrate for growth (Stahl et al., 2011). Thus, chemotaxis toward, adhesion to and subsequent utilization of

L-fucose by *C. jejuni* strains possessing a functional L-fucose uptake and metabolism pathway provides them with a competitive advantage. This seems, however, to be only the case during pathogenic (in human), but not during commensal (in chick) colonization (Stahl et al., 2011). Probably, next to decreasing the intestinal barrier permeability to *C. jejuni*, the highly sulfated fucosylated *O*-glycan mucin structures found in chickens decrease the accessibility of, and thus the responsiveness of *C. jejuni* to L-fucose. Indeed, upon feeding young chicks with an excess of free L-fucose also here a competitive colonization advantage was observed for wild-type *C. jejuni* over a mutant lacking a functional fucose permease gene, important for L-fucose transport into the bacterial cell (Stahl et al., 2011). Thus, a high degree of L-fucose masking through increased sulfation might give further explanation to the lack of association of *C. jejuni* with the chicken crypt epithelium *in vivo*. To conclude, there is increasing evidence that the composition of the chicken mucus layer is involved in the hindered contact between *C. jejuni* with the chicken intestinal epithelial surface. Indeed, *C. jejuni* is not closely associated with chicken crypt epithelium *in vivo* but rather resides in the mucus within the lumen of the crypts (Beery et al., 1988). However, the effect of chicken mucus on *C. jejuni* invasion in primary chicken epithelial cells has not yet been examined. Moreover, as the bacterium can be frequently detected in extra-intestinal organs of chicks, the mucus layer is not likely to be an efficient barrier to prevent close interaction with *C. jejuni* and the intestinal epithelial lining. In contrast, it seems that it indirectly promotes *C. jejuni* invasion through the secretion of Cia proteins (Biswas et al.,

2007). Further research will therefore have to reveal the genuine contribution of the mucus layer to GI and systemic colonization of *C. jejuni* in chicks.

Also the adaptive immune system of the chick might participate in the tolerogenic response to *C. jejuni*. Upon intestinal colonization, specific IgA against *C. jejuni* is induced. IgA is believed to induce the modulation of epitope expression by bacteria and to reduce intestinal proinflammatory signalling (Peterson et al., 2007). This indicates that the induction of IgA could lead to immune evasion, but whether the induction of IgA in chickens colonized with *C. jejuni* might be responsible for the noninflammatory *C. jejuni*-chicken gut relationship is not clear.

Next, murine intestinal epithelial cells are tolerized to LPS early after birth by exposure to exogenous LPS, facilitating microbial colonization and the establishement of a stable intestinal host-microbe homeostasis (MacPherson & Uhr, 2004). Whether in chickens LOS tolerance in the gut is involved in a tolerance-oriented integrated mucosal immune system, allowing commensal colonization of *C. jejuni*, is not clear.

Finally, chickens have an aberrant response to *C. jejuni* LOS and ~~are unresponsive to *C. jejuni*~~ flagellin, due to the absence of a late phase NF-κB response and TLR5 recognition sites, respectively. Only the first is likely to contribute to the differential *C. jejuni* response in humans and chicks because *C. jejuni* escapes TLR5 recognition in humans too (de Zoete et al., 2010). Next to these responses, colonized chickens might further induce tolerance by expressing factors that blunt *C. jejuni* components which could induce inflammation

(MacPherson & Uhr, 2004). However, potential candidates have not yet been identified.

Concluding remarks

Chickens are often colonized by the zoonotic pathogen *Campylobacter jejuni* and broiler meat products are considered to be the main source of campylobacteriosis in humans. In humans, *C. jejuni* is capable of causing severe inflammatory disease, while chickens are colonized asymptomatically. How *C. jejuni* shapes the mucosal immune system of the gut during health and disease is, however, poorly understood. Upon entering the chicken GI tract, *C. jejuni* establishes a complex interaction with its host, resulting in persistent high-level cecal colonization. Although evidence is emerging suggesting that *C. jejuni* poorly invades the GI tract of chicks and inefficiently elicits the chick's immune system, no pathology is observed. Moreover, it seems that *C. jejuni* is capable of evading the immune response and to even colonize systemically. This inefficient, controlled inflammatory response is not capable of clearing *C. jejuni* from the chicken gut and many processes might be involved in redirecting the response toward tolerance. The underlying mechanisms of the crosstalk between *C. jejuni* and chicks are just now starting to unravel and further research is warranted. Especially the mechanisms allowing this bacterium to persistently evade the immune response should deserve full attention. After all, a better understanding of the chick immune response upon *C. jejuni* entrance, as well as further elucidation

of the colonization mechanism of the bacterium in this host might promote the development of effective control measures to clear this human pathogen from poultry lines. For this purpose it might be of particular interest to identify chicken factors, if any, involved in blunting *C. jejuni* virulence factors, while *C. jejuni* colonization factors identified to da~~–~~te might hold promise for effective subunit vaccines. Moreover, the differential disease outcome in chicks and humans upon exposure to *C. jejuni* might be explained. Could it be due to the differences in mucin composition, TLR signalling, effect of CDT or humoral responses in these hosts, or are there other, yet to defined, mechanisms that ~~deside~~ determine ~~over~~ the commensal or pathogenic nature of *C. jejuni*. Answering these questions~~,~~ ~~based on what is currently known and described in this review,~~ could explain why and how a single bacterium is capable of causing severe inflammatory disease in one host while being (seemingly?) completely harmless in another.

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Declaration of Interest

The authors report no declarations of interest.

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