Campylobacter jejuni strategies to evade hostile environments

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Strategie van *Campylobacter jejuni* om te ontsnappen aan een vijandige omgeving

(met een samenvatting in het Nederlands)

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TO:

ESMAT, SORAYA, SOROUSH;

The love of my life

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GENERAL INTRODUCTION

1. *Campylobacter jejuni* infection

The Gram-negative bacterial species *Campylobacter* (1) is one of the most frequently isolated human food-borne pathogens (2, 3). Campylobacteriosis is a worldwide zoonotic disease affecting 0.1-1% of the European population each year (4, 5). The most frequent causative agent (>90%) of human *Campylobacter* infections is *Campylobacter jejuni* (*C. jejuni*). Although different domestic animals can be colonized with *Campylobacter* (6), the consumption of the chicken meat products is generally considered as the main source of human infection in the developed world (7, 8, 9). In European countries, around 80% of commercial flocks are colonized with *C. jejuni* (10). The ingestion of only 500-800 bacteria is already sufficient to cause an acute enterocolitis (11). Symptoms of infection include an acute watery or bloody diarrhoea, abdominal pain, fever, nausea, and headache (12, 13). The infection generally lasts for 1-3 weeks and then resolves naturally in most cases. In about 1 out of 1,000 cases, the infection may be followed by serious sequelae such as a reactive arthritis or neuroparalytic autoimmune disorders (e.g. Guillain-Barré syndrome). In cases of persistent human infection, antibiotic therapy may be indicated. This may be complicated by the increased dissemination of antibiotic resistant clones in animals and the human population (14-18).

Despite extensive efforts, there is no effective strategy to reduce the human incidence of *C. jejuni* infection. Major investments in educating the general public regarding the risks of consuming undercooked meat do not yield the desired results. A number of efforts aim to reduce chicken as a major reservoir of human infection with a focus on dietary measures, strict hygiene regimes, and technical innovations on poultry farms to limit animal transmission. Prevention of the colonization of chicken by vaccination is an attractive alternative approach. Trials to develop a *Campylobacter* vaccine for use in chicken show some progress but there is long far ahead to reach the final goal. Major problems are the development of cost-effective delivery methods, the induction of an effective immune response early in life, and the identification of cross-reactive protective antigens. Clearly, more knowledge about the commensal behaviour of *C. jejuni* in the chicken gut and the bacterial crosstalk with the chicken mucosal tissue is needed to make a major step forward in *C. jejuni* vaccine development.

2. *Campylobacter* pathogenesis

The reason why *C. jejuni* causes disease in humans and acts as a commensal in birds is still an enigma (19). Once ingested, *C. jejuni* is assumed to initially colonize the small bowel and then to move to the colon as its main niche of colonization or site of infection (20, 21, 22). These events seem to occur both in humans and in chicken (23, 24). In the human intestine, *C. jejuni* appears to penetrate the mucus layer and to survive in close proximity of the mucosal cells (25). Flagella driven motility and chemotaxis are crucial for *C. jejuni* to reach its favourable niche. The composition of the mucus layer in the chicken and human gut is quite diverse (26-28) but whether this influences *C. jejuni* pathogenicity is unknown. Genetic profiling of *C. jejuni* isolates indicates that the bacterium has adapted to a generalist lifestyle permitting rapid transmission between different hosts (29).

During human infection, large amounts of *C. jejuni* are present in intestinal crypts and seem to breach the epithelial barrier (30). Besides bacterial motility, no bacterial factors have been identified that are critical for this behaviour. *In vitro* experiments suggest that *C. jejuni* is capable to invade epithelial cells but also to pass the intercellular tight junctions to reach the sub-epithelial space (31, 32). Once present in the gut, *C. jejuni* is generally assumed to activate the innate immune system resulting in increased production of cytokine and chemokine production and the recruitment of inflammatory cells (33, 34, 35). The role of innate immune cells such as dendritic cells (DC), macrophages and monocytes in *C. jejuni* infection is uncertain (36, 37). It is evident that both colonization and clinical infection of the human gut elicit a humoral immune response. Immunodominant *C. jejuni* antigens are flagellins, lipooligosaccharide (LOS) and the major outer membrane porin (MOMP) (38, 39, 40, 41). It is generally accepted that frequent exposure of humans to different *C. jejuni* strains provides some protection against *C. jejuni* disease but not against intestinal colonization (42).

On commercial poultry farms, chicken are most susceptible for *C. jejuni* colonization at the first day and after 2-3 weeks of life. In the first few weeks after hatching, *C. jejuni* colonization is probably limited due to the presence of maternal immunity. The antigenic nature of the maternal IgA antibodies that provide protection against colonization is largely unknown but obviously may aid the discovery of potential vaccine antigens. Whether by absence of maternal immunity young chicken have a sufficiently mature immune system to respond to subunit vaccination is still a matter of debate.

After successful colonization, *C. jejuni* uses the chicken ceca as its main habitat. *C. jejuni* appears capable to penetrate the mucus layer of the chicken gut but the ability to breach the epithelial barrier seems to vary between strains and chicken breeds (43, 33, 34, 35). In general, colonization of chicken does not induce significant pathology but does generate a potent humoral antibody response. Bacterial challenge of chicken after attenuation of the immune system may indicate whether *C. jejuni* has the intrinsic ability to invade chicken or the chicken host defence limits the development of clinical pathology but such a study has not been performed.

3. Major surface characteristics of *Campylobacter jejuni*

Campylobacter belongs to the epsilon class of *proteobacteria* like the *Helicobacter* and *Wolinella* species (44). The genus *Campylobacter* consists of 17 species. *Campylobacter jejuni* and *Campylobacter coli* are the main members responsible for human infection. All *Campylobacter* genomes are relatively small and typically carry a large number of variable sequence regions that lead to considerable strain diversity (45). Most variable DNA regions among *C. jejuni* strains are loci encoding genes involved in the biosynthesis of capsule, LOS, and flagellins (46, 47, 48). In addition, single *C. jejuni* strains display considerable transcriptional phase variation enabling the strain to easily adapt their expression levels in response to changing environmental cues. The hyper variability of common traits of individual strains and among *C. jejuni* strains complicates the understanding of the bacterial pathogenesis and the development of an effective vaccine.

Capsule

It has long been assumed that *C. jejuni* does not produce a polysaccharide capsule at its outer surface. The first indication to *C. jejuni* may be able to produce such a capsule was after the identification of a previously unknown gene cluster within the *C. jejuni* genome. Subsequent targeted mutagenesis and biochemical and morphological analyses showed that the gene cluster indeed enabled capsule biosynthesis. It is now well established that the *C. jejuni* capsule plays an important role in bacterial adhesion, invasion, chicken colonization and virulence (49, 50), although the exact mechanisms remain to be defined. The capsular polysaccharides of different *C. jejuni* strains can vary in composition and the linkages of the sugars. This diversity is at the basis of the Penner serotyping scheme (51). Besides

differences in the carbohydrate backbone, the capsule of some strains displays variation via heptoses with an unusual O-methyl phosphoramidate linkage (MeOPN). This modification has a significant effect on the bacterial adhesion to and invasion of host cells (52, 53). Furthermore, reversible phase variation of the capsular structures can modulate phage infectivity and lead to continuous phage-host co-evolution in the chicken gut (54, 55). As for most bacterial pathogens, the capsule of *C. jejuni* has an important role in the protection against the bactericidal effects of serum and the shielding of surface antigens (56). The biological significance of the observed regulation of capsule expression during different environmental conditions is unknown but does influence the function of the type VI secretion system that is present in some *C. jejuni* strains (57). As the capsule polysaccharide is immunogenic during human infection, capsular structures are under investigation as future human vaccine targets.

Lipo-oligosaccharide (LOS)

As a Gram-negative microorganism, the main constituent of the bacterial outer membrane is lipopolysaccharide (LPS). As the glycan chain of this bacterial glycolipid is rather short and lacks the O-antigen typically produced in many enteropathogens, the *C. jejuni* LPS is often referred to as lipo-oligosaccharide (LOS). The LOS of *C. jejuni* is composed of a hexa-acylated lipid A that is anchored in the outer membrane and linked to a highly variable surface-exposed carbohydrate moiety. The variation in LOS between *C. jejuni* strains is caused by the diverse repertoire of LOS biosynthesis genes (58). In addition, LOS may display intra-strain variation due to the presence of homopolymeric tracts within the promoter or coding regions of distinct genes (59). The role of LOS variation in the natural infection is unknown but it may act as an immune escape mechanism. Alternatively, the different OS may target different host surface lectin receptors and thus modulate the host response. Immunogenic analysis of the LOS structures has demonstrated that certain OS structures of *C. jejuni* resemble those on host cell glycolipids, limiting their use as vaccine antigens. The molecular mimicry of certain LOS species with neuronal gangliosides explains the pathogenic role of *C. jejuni* LOS in the development of anti-antibodies that cause the autoimmune syndromes that may follow *C. jejuni* infection (60-64).

Glycosylated flagella

C. jejuni is a highly motile bacterium. The presence of a single flagellum at each pole together with its typical spiral-shape enable the pathogen to actively penetrate viscous environments such the intestinal mucus layer. The flagellum of *C. jejuni* follows the overall bacterial flagellum architecture. It consists of a basal body spanning the bacterial membranes, a hook filament, and a long flagella fibre that protrudes from the outer surface. *C. jejuni* produces two major structural flagellins (FlaA and FlaB) as flagella subunit proteins. One flagellum consists of thousands of flagellin subunits that grow into a filament at the distal FliD capping complex. Although the overall flagellum structure is rather conserved, the protein sequence of flagellins of different strains is quite diverse (65), resulting in antigenic heterogeneity. In contrast to the flagellins of most other enteropathogens, the flagellins of *C. jejuni* evade recognition by the innate immune receptor Toll-like receptor 5 (TLR5) (66, 67).

Besides diversity in protein sequence, the surface-exposed regions of *C. jejuni* flagellins are decorated with different carbohydrates at serine or threonine residues. The machinery to attach *O*-glycans to the *C. jejuni* flagellins is encoded by a large cluster of genes that displays variation between strains resulting in additional antigenic diversity (68). The presence of phase variable tandem repeats within distinct gene promoters or coding regions of the *O*-glycosylation locus may cause additional intra strain glycan variation. A single flagellin may contain up to 20 different glycosylation sites.

In general *C. jejuni* motility is regarded as crucial for bacterial colonization of the chicken gut and host cell infection (69). The flagella are highly immunogenic and antibodies directed against the flagella are considered to protect against infection with the homologous strain (70). The function of glycosylation of the flagella is unknown but the event is essential for flagella assembly (71) and the glycans may act as an attachment site for distinct bacteriophages. It is assumed that the flagella sugar coat also facilitates the movement of the bacterium through the mucus. The potential of *O*-linked glycans (that mainly consist of sialic acid derivatives) as targets to prevent colonization or infection is largely unknown. This is contrast to the *N*-linked glycans that *C. jejuni* attaches to asparagine residues on many membrane proteins. These glycans are under investigation as a conserved vaccine antigen target (72, 73, 74).

***Major surface protein antigens of* C. jejuni**

Besides glycolipids and glycoproteins, *C. jejuni* carries a number of outer membrane proteins. Well-characterized proteins that are largely conserved among *C. jejuni* strains include the major outer membrane porin (MOMP), several putative adhesions, as well as proteins involved in iron acquisition. The *Campylobacter* adhesion to fibronectin (CadF) protein promotes bacterial adhesion through binding to fibronectin on epithelial cells (75). CadF also facilitates the colonization of chicken gut (76). The biological significance of the putative adhesions JIpA (77), CapA, Peb1 (78), the glycoprotein Cj1496c (79) and FlpA are much less well defined and are still under debate (77-81). *C. jejuni* lacks fimbriae or typical type III or type IV protein secretion systems as are present in many enteropathogens. Secretion of bacterial proteins into the environment largely occurs through the flagellar export apparatus (82, 83). Upon co-cultivation of *C. jejuni* with intestinal cells a subset of *de novo* synthesized proteins, termed Cia proteins are secreted through the flagellar secretion machinery (84). Some Cia proteins are delivered to cytosol of host cells and may affect the intracellular fate of the bacterium (85) but this needs more confirmation. The role of Cia proteins in chicken colonization or human infection, remains to be elucidated.

C. jejuni does not produce potent cytotoxins. The best-characterized toxin is the cytolethal distending toxin (CDT). This toxin displays DNAse activity and induces growth arrest at the G2/M stage in eukaryotic cells (86, 87). When orally administered to SCID mice a CDT-defective strain is unaffected in enteric colonization but shows impaired systemic dissemination (88). However, the CDT toxin is not present in the genome of all disease isolates and thus does seems not essential for the establishment of an infection. A small subset (about 10%) of *C. jejuni* strains expresses a Type VI secretion system that causes capsule-sensitive cytotoxicity towards red blood cells. This feature may also be associated with more invasive disease (57) but this needs further study.

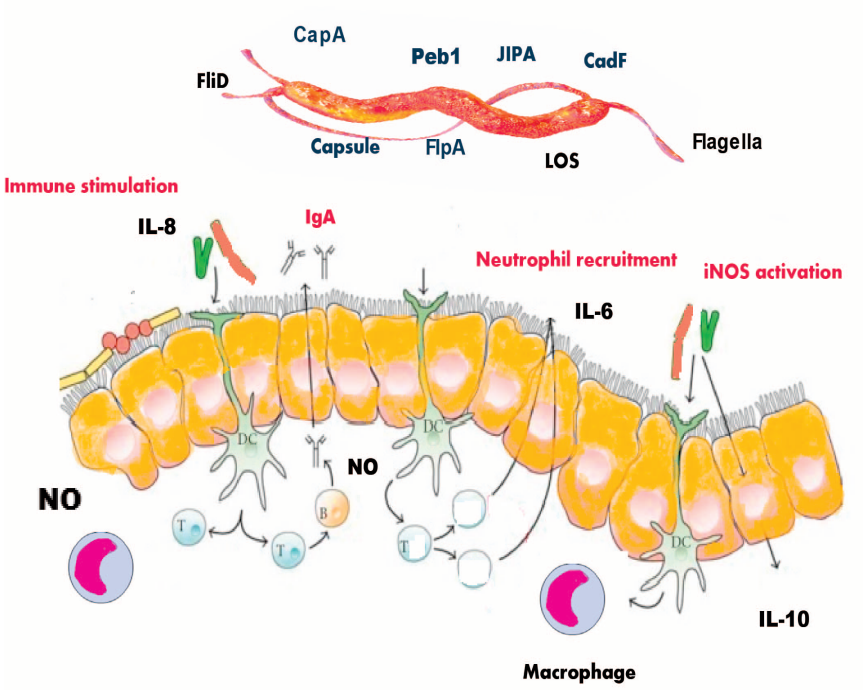

Figure 1. Major surface structures of *C. jejuni* and the induced host response. *C.jejuni* **capsule** may contribute to bacterial adhesion/invasion and the colonization of chicken. **Lipo-oligosaccharide (LOS)** probably acts as an immune scape mechanism. **CDT** toxin exhibits DNAse activity and induces growth arrest at the G2/M stage in eukaryotic cells. **JIpA, Peb1, FLpA,** and **Cia** may have adhesive functions. **CadF** facilitates colonization and bacterial adhesion. **Flagella** are responsible for motility and immunogenicity and acts as a bacterial protein secretion system. The host response induced by *C. jejuni* includes the production of chemokines, cytokines, immunoglobulins like IgA, and the recruitment of innate immune cells.

4. *Campylobacter* metabolism

The failure to identify obvious virulence determinants has boosted awareness that *C. jejuni* may have evolved non-classical virulence strategies. One molecular interaction that is gaining attention is the metabolic crosstalk between *C. jejuni* and the host. In comparison to most other enteropathogens *C. jejuni* is exceptional in that it utilizes distinct amino acids rather than glucose as a primary energy source (89) and favours a microaerophilic atmosphere. At the host mucosa, *C. jejuni* may utilize host cell nutrients and thus influence host cell biology and immunometabolism (90, 91, 92). This concept is supported by the fact that although *C. jejuni* is incapable to metabolize glucose due to the absence of the enzymes

glucokinase (46) and phosphofructokinase (93), some strains are able to utilize L-fucose (94, 95) which is a major component of mucus (96, 97, 98). Furthermore, the preferred amino acids utilized by *C. jejuni* are serine, aspartate, glutamate, and proline (99, 100, 101) which are the most abundant amino acids in the chicken cecum (102, 103, 104). Some strains can also utilize asparagine and glutamine (105, 106). The respiratory nitrite denitrification pathway of *C. jejuni* plays an important role in resisting and using host cell-derived nitrosative compounds and enables optimal adaptation to low oxygen environments. To assess the significance of the metabolic interplay between *C. jejuni* and the host mucosa, detailed knowledge of *C. jejuni* metabolism is needed.

Serine

Serine is one of the best-known chemoattractants that can be sensed by *C. jejuni* (107, 108). Serine metabolism is largely determined by the activities of the SdaC serine transporter protein (Cj1624c) and the SdaA L-serine dehydratase (Cj1625c). SdaA catalyzes the deamination of serine to pyruvate and ammonia. Pyruvate is a major carbon source of *C. jejuni* (109). Ammonia may serve as a nitrogen source but may have additional functions that deserve more study. The SdaA enzyme is an oxygen sensitive iron–sulfur complex and this is probably one of the reasons of the poor oxygen tolerance of the bacterium (106, 107). The SdaA protein is essential for *C. jejuni* colonization of the chicken cecum consistent with the assumed key role of serine as bacterial nutrient in the chicken gut.

Aspartate/glutamate

Aspartate is taken up by *C. jejuni* through the Peb1 system (110) or the C4-dicarboxylate transporters (DctA, DcuA, and DcuB). After uptake, the aspartate is deaminated by the aspartase enzyme AspA /B (111). Deamination by AspA results in the formation of fumarate and ammonia. The fumarate can enter the citric acid cycle (112), while (as mentioned above) the fate of ammonia needs more study. The methyl-accepting chemotactic protein (MCP, Tlp1) has been identified as an aspartate receptor of *Campylobacter* (113). The importance of aspartate utilization in intestinal colonization is apparent from the altered bacterial motility and increased invasion of human intestinal cells after genetic inactivation of the *tlp1* gene and the reduced ability of the mutant strain to colonize chickens (114, 115).

The amino acid glutamate is also transported *via* the Peb1 system (110). Glutamate is transaminated by AspB to aspartate and 2-oxoglutarate. The aspartate is deaminated by AspA into fumarate and ammonia. The fumarate and 2-oxoglutarate are fed into the citric acid cycle (116, 117, 118). This design of the glutamate pathway implies that AspA is a key intermediate in both the utilization of aspartate as well as glutamate.

Proline

The uptake of proline occurs by the PutP transporter and the enzyme PutA (101). The PutA enzyme of *C. jejuni* is predicted to have both proline dehydrogenase and delta-1-pyrroline-5-carboxylate dehydrogenase activities with the use FAD and NADH as cofactors, respectively. The PutA protein facilitates the oxidation of proline to glutamate. As glutamate is transaminated to aspartate, the utilization of proline also requires AspA enzyme activity. Whether bacterial proline utilization influences, for instance, the degradation of the proline-rich extracellular matrix (ECM) by the proline-deprived host cells, is unknown.

Asparagine / glutamine

The ability of most *C. jejuni* strains to utilize the amino acid asparagine depends on the secretion of the enzyme asparaginase encoded by the *ansB* gene. The enzyme deaminates asparagine to aspartate in the periplasm, preventing the need for a separate asparagine transporter protein. The fate of the produced ammonia has not been investigated (119). The ability to utilize asparagine is not critical for the colonization of the chicken cecum by *C. jejuni* (120). In eukaryotic cells (and in *C. jejuni*) asparagine-linked glycosylation plays an important role in a range of cellular processes (121, 122) but whether bacterial asparagine utilization influences these processes remains to be determined.

C. jejuni can import glutamine via the Paq transporter system (101). Efficient use of glutamine for growth however, occurs after periplasmic hydrolysis of glutamine to glutamate and ammonia via the enzyme γ-glutamyltranspeptidase (GGT) (123, 124, 125). This enzyme is present in 30-35% of *C. jejuni* isolates, suggesting that it is not essential to establish human infection. The presence of GGT provides *C. jejuni* strains a survival advantage in the presence of bactericidal isothiocyanates (126). Whether the enzyme influence the mucus layer or cell metabolism of the host cells has not been investigated.

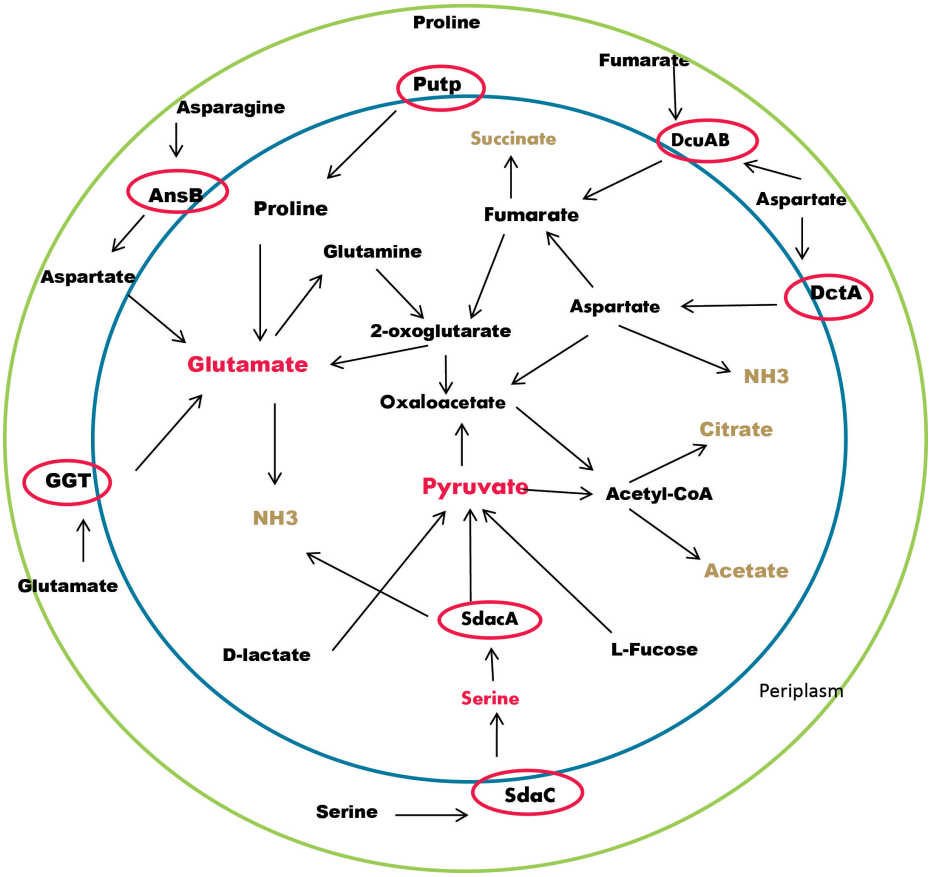

Figure 2. Overview of the main amino acid transporters and metabolic pathways of *C. jejuni*. Pyruvate and glutamate play a central role in *C. jejuni* metabolism. While L-serine, lactate and L-fucose metabolism results in pyruvate production, asparagine, aspartate and glutamine metabolism results in glutamate production. In both metabolic pathways ammonium is released as the waste product. Amino acid transporters are encircled.

Nitrate-nitrite-ammonia pathway

In order to generate the proton gradient needed for energy production, *C. jejuni* has evolved a complex branched electron transport chain that enables the use of an array of combinations of electron donors and acceptors. During microaerophilic conditions, the final acceptor of electrons is molecular oxygen. At very low oxygen conditions as likely exist in the colon; alternative molecules are used such as nitrate (127, 128, 129). *C. jejuni* uses the periplasmic nitrate reductase system (Nap) to catalyze nitrate reduction, whereas for

reduction of nitrite to ammonia (NH_3) the Nrf system is available (130, 131). The biological fate and function of the ammonia produced by the respiratory denitrification pathway is largely unknown.

Apart from utilizing amino acids and electron donors and acceptors from the host environment, the colonizing *C. jejuni* is exposed to potential harmful oxidative and nitrosative stress. *C. jejuni* is equipped with several mechanisms that neutralize these stress conditions (132) but whether these strategies disturb host cell biology and contribute to the infection, is unknown.

5. Aim and outline of this thesis

C. jejuni remains the most frequent cause of human foodborne infections causing worldwide tens of millions of cases of enterocolitis each year. **Chapter 1** of this thesis gives an overview of the main characteristics of *C. jejuni* behaviour and disease. This shows that despite its broad impact on human health, the bacterial mechanisms that contribute to *C. jejuni* colonization and symptomatic infection are still an enigma. This lack of knowledge seriously hampers the development of efficient strategies to control *C. jejuni* transmission and the prevention of colonization and infection. The aim of the work described in this thesis is to answer burning questions in the *C. jejuni* research field. These issues cover the broad spectrum of possible strategies of vaccine delivery to young chicken up to the unraveling of the molecular crosstalk between *C. jejuni* and the host environment in order to identify new protection targets.

The specific questions addressed in this thesis are:

a. Is the chicken immune system at the late embryonic stage sufficient mature to use *in ovo* vaccine technology to deliver bacterial subunit vaccines?

b. Is the commensal behaviour of *C. jejuni* in chicken due to an intrinsic lack of bacterial virulence in the chicken and/or the quality of the chicken host defence to limit *C. jejuni* infection?

c. How is *C. jejuni* capable to survive and actively shape its own microenvironmental niche in the complex intestinal habitat of the chicken gut?

d. Does metabolic interplay between *C. jejuni* and the host immune system contribute to bacterial colonization and disease?

Chapter 2 focuses on the possible application of *in ovo* immunization technology to protect chicken against bacteria at a very young age. *In ovo* vaccination has thus far barely been applied for bacterial vaccines. This is partially due to the fundamental issue of whether the immune system at the late embryonal stage is sufficiently mature to provide the desired immune response. As described in **Chapter 2**, we injected 18-days old chicken embryos with purified flagellin protein of *Salmonella* and followed the development of the innate and adaptive immune response up to 2 weeks after hatch. We used *Salmonella* FliC as proof-of-principle antigen because this protein has intrinsic adjuvant (TLR5-stimulating) activity and because a putative vaccine composed of recombinant *C. jejuni* flagellin with intrinsic TLR5-stimulating activity is under development. The development of an innate immune response and of specific antibodies would indicate that *in ovo* vaccination is a feasible strategy to reduce *C. jejuni* colonization in chicken.

The intriguing issue as to why the human pathogen *C. jejuni* exhibits commensal behaviour in gut of chicken is addressed in **Chapter 3**. The chicken-or-egg dilemma here is whether in the environment of the chicken gut *C. jejuni* lacks critical virulence determinants and/or whether the chicken immune system limits the manifestation of pathology. To resolve this issue, we followed the colonization and organ dissemination of *C. jejuni* in healthy chicken and in immuno-compromised animals. In these experiments, challenge of the animals with *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) was used as a comparison. Enhanced dissemination of the bacteria in the immuno-comprised animals would imply that the host immune defence is able to limit the infection but also that exposure of the chicken to stressful conditions may increase the risk of bacterial dissemination from the gut to distant organs.

To better understand how *C. jejuni* is able to colonize the chicken gut in such high quantities, we investigated how the bacterium may shape its local environmental niche to allow this outgrowth. As described in **Chapter 4**, we focused our studies on the bacterial utilization of different carbon and energy sources and their effect on the environmental pH. In these studies, we specifically compared the effects of amino acid and carbohydate metabolism of *C. jejuni*, *E. coli* and *S.* Enteritidis. Knowledge of the *C. jejuni* metabolic

strategies to manipulate the gut ecosystem may potentially provide novel opportunities to limit the colonization by targeted redirection of metabolic behaviour.

Awareness is increasing that metabolic cross talk between bacteria and the host mucosa are an important in both intestinal homeostasis and development of disease. In **Chapter 5,** we focused on the potential function of *C. jejuni* metabolism as a regulator of the innate immune response. We infected chicken macrophages with *C. jejuni* and then determined cytokine and chemokine transcript levels, the production and bacterial utilization of nitric oxide, and the S-nitrosylation status of the host cells. This work should indicate whether *C. jejuni* exploits its metabolism to alter the host immune response and thus whether bacterial metabolism may be a potential target to combat the zoonotic pathogen.

The major findings and potential of the work described in this thesis are summarized and discussed in **Chapter 6**.

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Chapter 2

Chicken Immune Response Following *in ovo* Delivery of Bacterial Flagellin

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ABSTRACT

***In ovo* immunization of chicken embryos with live vaccines is an effective strategy to protect chicken against several viral pathogens. We investigated the immune response of chicken embryos to purified recombinant protein. *In ovo* delivery of *Salmonella* flagellin (FliC) to 18-day-old embryonated eggs resulted in elevated pro-inflammatory chIL-6 and chIL-8 (CXCL8-CXCLi2) cytokine transcript levels in the intestine but not in the spleen at 24 h post-injection. Analysis of the chicken Toll-like receptor (TLR) repertoire in 18-day-old embryos revealed gene transcripts in the intestinal and spleen tissue for most chicken TLRs, including TLR5 which recognizes *Salmonella* flagellin (FliC). The *in ovo* administration of FliC did not alter TLR transcript levels, except for an increase in intestinal chTLR15 expression. Measurement of the antibody response in sera collected at Day-11 and Day-21 post-hatch demonstrated high titers of FliC-specific antibodies for the animals immunized at the late-embryonic stage in contrast to the mock-treated controls. The successful *in ovo* immunization with purified bacterial antigen indicates that the immune system of the chicken embryo is sufficiently mature to yield a strong humoral immune response after single exposure to purified protein. This finding strengthens the basis for the development of *in ovo* protein-based subunit vaccines.**

INTRODUCTION

Protection of chicken against bacterial and viral pathogens is important for animal and human health. An effective and convenient protection strategy is active immunization of embryonated eggs (1, 2, 3, 4). During this procedure, the vaccine is injected into the amnion sac or intramuscularly into the chicken embryo usually at 3 days prior to hatch i.e., at Day-18 of embryonic development (ED18) (5). The immunization evokes antibodies directed against the vaccine antigens, resulting in protection early after hatching. *In ovo* vaccination is commercially widely used to protect against viral infections. Most licensed vaccines consist of live attenuated viruses that can still replicate and provoke an immune response, but do not cause illness (6). More recently, non-replicating adenovirus-vector based vaccines have been developed (7, 8). *In ovo* delivery of subunit vaccines that consist of a mixture of purified antigens rather than of live vaccine carriers, is still in its infancy. Successful *in ovo* immunization has been achieved with recombinant *Eimeria* protein (9, 10, 11) but immunization with a recombinant protein of *Campylobacter jejuni* failed to induce a significant immune response (12). The reason for the apparent variable immune response to recombinant bacterial proteins after *in ovo* delivery is unknown.

One factor that aids the generation of a potent immune response upon immunization is the use of vaccine adjuvants or other immunomodulatory agents such as distinct cytokines. These compounds promote the immunogenicity of vaccine antigens and influence the quality of the adaptive immune response (13, 14, 15). The repertoire of potential adjuvants for use in chickens was boosted by the discovery of functional chicken Toll-like receptors (TLR) (16, 17). Members of the TLR family of pathogen recognition receptor sense microbial ligands and translate these signals into pro-inflammatory signals that promote amongst others antigen presentation by dendritic cells, and T- and B-cell responses (18, 19, 20) TLR agonists are beginning to be applied as vaccine adjuvants (21, 22), also in the chicken (23). The effect of TLR stimulation on the immune response seems most effective when the antigen of interest has intrinsic TLR-stimulating activity or is conjugated to an effective TLR agonist.

TLR ligands that are investigated as adjuvants in chickens include mainly flagellin (24, 25) and CpG oligodeoxynucleotides (10, 26, 27).These compounds target chTLR5 and chTLR21 receptors, respectively (28, 29, 30, 31). A prerequisite for the use of TLR agonists as an adjuvant in combination with *in ovo* vaccination is the expression and

function of the relevant TLR receptors at the late embryonic stage. Transcriptional profiling of immune genes during chicken embryo development indicates early but variable presence of TLR transcripts throughout the embryonic development (32, 33, 34).

In the present study we investigated the expression of TLR genes at the day of *in-ovo* vaccination and the effect of *in ovo* delivery of a recombinant bacterial antigen with intrinsic TLR5 stimulating activity on the generation and duration of an antigen-specific humoral immune response. We provide evidence that a single-dose injection of recombinant *Salmonella* flagellin into the amnion sac of chicken embryos results in an intestinal cytokine response and the induction of specific IgY antibodies that can easily be detected up to 21 days post-hatch.

MATERIALS AND METHODS

Construction, Expression, and Purification of Recombinant Salmonella His-tagged Flagellin

Recombinant flagellin (FliC) of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) *was* produced as previously described (35) with some minor modifications. Briefly, the *fliC* gene of *S.* Enteritidis strain 90-13-706 was amplified by PCR, cloned with an N-terminal 6xHis-tag into the pT7.7 protein expression vector, and transformed into E.*coli* BL21 star (DE3). Protein expression was induced by adding 1 mM of IPTG to bacteria (OD_{550} of 0.4) grown (37^oC) in LB broth containing 100 µg/ml of ampicillin. Four hours after induction, bacteria were harvested by centrifugation and resuspended into urea solution (8 M urea in 100 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8). After 16 h of incubation (20^oC, constant rotation), insoluble materials were removed by centrifugation. The supernatant containing the FliC protein was mixed (1 h) with Ni-NTA beads (Qiagen). After washing of the beads with 4x4 ml of 8 M urea solution with pH 6.3, bound FliC protein was eluted with 4x0.5 ml of 8 M urea solution with pH 5.9 and, subsequently, with 4x0.5 ml of 8 M urea solution with pH 4.5. The FliC containing fractions were determined by 12.5% SDS-PAGE, pooled and stored in 4 M urea solution. Protein concentrations were measured with the Pierce BCA protein assay kit.

Animal Experiments

Fertilized eggs from SPF (Ross 308) broilers (Gezondheidsdienst, Deventer, the Netherlands) were kept at 38^{o}C and 65-75% relative humidity in a forced air egg incubator. At Day 18, the eggs were candled to check their fertilization, and then divided into three groups of 15 eggs. Group 1 received 20 µg of FliC protein diluted in 100 µl of 10 mM Tris (pH 9.0) / 20% glycerol / 5 mM sucrose / 80 mM urea (FliC group). Embryonated eggs of group 2 were injected with 100 µl of the same solution lacking FliC protein (mock group). The eggs of group 3 were kept intact and received no treatment (non-injected group). For *in ovo* delivery we followed the procedure described by Sharma [1]. Briefly, after cleaning the eggs with 0.5% hypochlorite (bleach), a small hole was made at the air sac end of the egg using an 18G sterile needle. A 22G one-inch bevel needle (Monoject) was then used to deliver 20 µg of flagellin (or solvent) through the air sack membrane directly into the amnion fluid. After 24 h of incubation, the embryos of five eggs from each group were aseptically removed to isolate the gut and spleen tissue. Organ samples were immediately frozen in liquid nitrogen and stored at -80 until further analysis. The remaining eggs were kept in the incubator until hatch. After hatch, the chickens were reared in a ground stable under controlled hygienic conditions for up to 21 days. Chickens were given access to water and commercial broiler diet *ad libitum* without antibiotics or coccidiostats. At Day-11 post-hatch, blood samples were taken from the wing vein for antibody analysis. At Day-21 post-hatch, all chickens were sacrificed by electrocution and blood was collected. After blood clotting and centrifugation (2,000 x *g*, 5 min, 4°C), sera were collected and stored at -20°C until analyzed. The entire experiment was repeated in the same setup with eggs from a commercial (non-SPF) flock (Lagerweij, Lunteren, the Netherlands). The *in ovo* immunization procedure did not influence the hatchability and chicken survival. All experiments were conducted in accordance with protocols approved by the Dutch experimental animal committee (DEC).

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from 50 (±5) mg of the collected embryonic tissue specimen. Samples were homogenized (6,500 x g for 50 s at 4^{o}C) in a MagNA Lyser instrument (Roche) using Lysing Matrix D tubes (MPbio) filled with 1 ml of RNA-Bee (Bio-connect USA). Total RNA was extracted using the RNA-Bee isolation kit according to the instructions of the manufacturer. The quantity and purity of the extracted RNA was

measured at 260/280nm in a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). After treatment with DNAse (1 U/µg of RNA, Fermentas), one microgram of RNA was reverse transcribed to cDNA using the Revert Aid^{TM} First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Transcripts of chicken *TLR* genes and (as control) the chicken *GAPDH* gene (*chGAPDH*), were determined by PCR using the primers listed in Table 1. In all cases, RT-negative control samples were run to verify the absence of contaminating DNA. PCR amplification was performed using 1 µl of cDNA, 200 nM of each primer, 1 mM of dNTPs, and 1 Unit of Taq DNA polymerase (Fermentas) in a total reaction volume of 20 µl. The following cycle conditions were used: one initialization step at 95^{o}C for 5 min, 35 cycles of denaturation at 95^{o}C for 30 s, annealing at 62^{o}C for 30 s, and extension at 72^{o}C for 35 s, followed by one cycle at 72^{o}C (10 min). RT-PCR products were resolved by electrophoresis using 2% TBE agarose gels stained with ethidium bromide and imaged under UV illumination (Pharmacia Biotech). Results shown are representative of one of two replicates and with cDNA isolated from the indicated tissues of five embryos of 19-day-old chicken.

Table 1. Chicken TLRs primers used in this study.

Name	Sequence
chTLR1b	AGAAAAGGCTCCAGGCTACGA TACGACGTTCCCCAGTTGTGT
chTLR2t1	TGAAGCTGATGTGGAAGCA ACACCGTGATTTTGCCTGTGA
cTLR2t2	GATGCCAGCGCCAATAACTTTA TCCATCAGTGACAGCTGCACA
chTLR3	GCACCTGTGAAAGCATTGCTT AATGGAGCACTGTCCTTGCA
chTLR4	CCTGAAATGGGTCAAGGAAAAG CTGTGGTTGGGTTGGGATG
chTLR5	GAAATTGGAACACAACGCGTC CGGAGTATGGTCAAACGTTGC
chTLR7	GGCTGTGAATGAATGAATGGGTGA GCTGAATGCTCTGGGAAAGG
chTLR15	GAAAATAAGCCCTTCGATGCCT TGTTGCCAAGTAACAGGATGCC
chTLR16	TTGCTTGCACGTCTTCGACAT TTAGGAAGACCGTGTCCAGGTG
chTLR21	GCAGCTAGCCGCTCCTTTT CCTTCTTCTTCCTCCTCCTCTCC
chGAPDH	TGCTGTGCTGGCCATCTTT GCTTTGGACGTGCTCCAAA

Quantitative Real-time PCR Analysis

Cytokine mRNA levels in the isolated tissue samples were measured by RT-qPCR as described previously (36). Briefly, RT-qPCR was performed using the Reverse Transcriptase qRT-PCR Master Mix-Kit (Erurogentec, Seraing, Belgium). For each condition, 50 ng of DNaseI (Fermentas) treated RNA was used as template. The sets of primers and probes that were used are listed in Table 2. The amplification reactions were performed in a Roche LightCycler® 480 using the following conditions: Reverse Transcription step at 48°C (30 min), followed by 10 min at 95°C, 40 sequential cycles of 15 s at 95°C and 1 min at 60°C. Each sample was run in duplicate. Transcript levels were normalized to those for the chicken housekeeping gene *chGAPDH*. For each gene, results were expressed as fold change in mRNA level of the immunized embryos compared to the

non-injected controls according to Schmittgen & Livak (37) using the formula: (1) ΔC_t target gene - ΔC_t GAPDH for each sample, (2) ΔC_t target gene treated - ΔC_t target gene control. The fold change in mRNA for each gene transcript was determined using the formula: Fold change = $2^{-\Delta (\Delta \text{ Ct gene treated } -\Delta \text{ Ct gene control})}$. To calculate the relative expression levels for FliC- and mock-treated, the transcript levels in samples of individual embryos were compared with the mean value of the group of non-injected embryonated eggs, yielding a mean ± SEM value for the mock-treated and FliC-injected groups. The SEM values thus represent the variation in fold difference between individual chicken.

Table 2. Primers and probes used in this study.

GAPDH	Forward Reverse Probe	GCCGTCCTCTCTGGCAAAG TGTAAACCATGTAGTTCAGATCGATGA (FAM)-AGTGGTGGCCATCAATGATCC-(TAMRA)
IL-8	Forward Reverse Probe	GCCCTCCTCCGGTTTCAG CGCAGCTCATTCCCCATCT (FAM)-TGCTCTGTCGCAAGGTAGGACGCTG(TAMRA)
IL1-ß	Forward Reverse probe	GCTCTACATGTCGTGTGTGATGAG TGTCGATGTCCCGCATGA (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)
INF-β	Forward Reverse probe	ACAACTTCCTACAGCACAACAACTA GCCTGGAGGCGGGACATG (FAM)-TCCCAGGTACAAGCACTG-(TAMRA)
IL-6	Forward Reverse probe	GCTCGCCGGCTTCGA GGTAGGTCTGAAAGGCGAACAG (FAM)AGGAGAAATGCCTGACGAAGCTCTCCA (TAMRA)

Cell Culture, Transfection, and Gene Reporter Assay

The HeLa-57A cell line, stably transfected with a NF-κB luciferase reporter construct (38)was generously provided by Dr. R. T. Hay (Institute of Biomolecular Sciences, University of St. Andrews, St. Andrews, Scotland, U.K.). Cells were routinely propagated in 25-cm^2 tissue culture flasks (Corning) in DMEM with 10% FCS at 37°C in a 5% CO_2 atmosphere.

For transfection, cells were propagated in 24-well tissue culture plates in DMEM with 10% FCS until 70% confluence was reached (~24 h). Then, cells were transiently transfected with plasmid pTracer 3xFLAG-chtlr5 or (as control) empty vector, in DMEM

without FCS using FuGENE HD (Roche Diagnostics) at a lipid to DNA ratio of 3 to 1, as previously described (29). After 4 h of incubation (37°C), the medium was replaced with fresh medium containing DMEM with 10% FCS. Functional assays were performed at 48 h post-transfection.

ChTLR5 signaling was essentially assessed as described (39). In brief, transfected cells were stimulated with 10 ng ml^{-1} of *Salmonella* flagellin (or solvent) for 5 h, rinsed with PBS, and immediately lysed in reporter lysis buffer (Promega). Firefly luciferase activity was measured with a luciferase assay system (Promega) using a luminometer (TD-20/20, Turner Designs).

Measurement of FliC-specific IgY Antibodies

Flagellin-specific IgY antibodies were detected using Maxisorb 96-wells ELISA plates coated (16 h, 4^{o}C) with 2.5 µg ml^{-1} of FliC protein in carbonate-bicarbonate buffer. Plates were washed 5 times with PBS-T (10 mM phosphate buffer, 150 mM NaCl, 0.05% Tween 20, pH 7.4), blocked (2 h) with 5% FCS diluted in PBS, and incubated (1 h, 20^{o}C, constant shaking) with serial dilutions of chicken sera in assay buffer (5% FCS, 0.5%Tween 20 in Tris-buffered saline). After rinsing of the wells with PBS-T, goat-anti-chicken IgY-Fc antibody conjugated to horse reddish peroxidase (HRP) (AAI29P, AbD Serotec, dilution: 1:2,500 in assay buffer) was added. After 1 h of incubation, the wells were rinsed with PBS-T and HRP substrate (3,3',5,5'-Tetramethylbenzidine, TMB) was added. After 10 min of incubation (in the dark), the enzyme reaction was stopped by adding H_2SO_4. Absorption (450 nm) was measured using a Fluostar Omega spectrophotometer. Antibody titers for the sera of each of the hatched chickens were defined as the highest sera dilution giving statistically significant differences between the immunized and control group.

Statistical Analysis

GraphPad Prism 6.05 software was used for all statistical analyses. Data were analyzed by multiple t-test for comparison of two groups using the Holm-Šidák method. Differences were considered to be statistically significant at $P < 0.05$.

Ethics Statement

All procedures involving animals were conducted in accordance with protocols approved by the Dutch experimental animal committee (DEC).

RESULTS

In Ovo *Delivery of Molecules*

To investigate the fate of recombinant protein delivered into the amniotic fluid, we first monitored the tissue distribution of Patent Blue V (2.5%) after injection into 18-day old embryonated eggs. Macroscopic examination of the tissues of the embryos at Day-1 post-injection revealed strong staining of the distal part of the intestine but not of other organs of the embryo. This suggested that the injected material was ingested and accumulated mainly in the chicken gut. In these experiments, the length of the needle (22-gauge) that was used for *in ovo* delivery into the amniotic sac was critical as injection with needles of different length resulted in local staining of the embryo at the injection site without accumulation of the stain in the chicken gut.

Expression of Toll-like Receptors in the Embryonic Gut

To enable a rational choice of a TLR agonist as *in ovo* vaccine adjuvant, we next investigated the expression of TLRs in gut and spleen tissue isolated from non-injected 19-day-old embryonated eggs. RT-PCR transcript analysis on extracted tissue RNA demonstrated that both organ tissues expressed chTLR1b, chTLR2t1, chTLR2t2, chTLR3, chTLR4, chTLR5, chTLR7, and chTLR21 (Figure 1). ChTLR15 and chTLR16 (TLR1a) appeared predominantly expressed in spleen tissue but weakly expressed in gut tissue (Figure 1). All RNA samples used for cDNA synthesis were demonstrated to be free from genomic DNA contamination by the absence of transcripts in the non-reverse transcribed samples data available (data not shown). Together, the TLR expression profiles suggest that most TLR receptors are expressed at the main intestinal antigen delivery site in 19-day-old embryos.

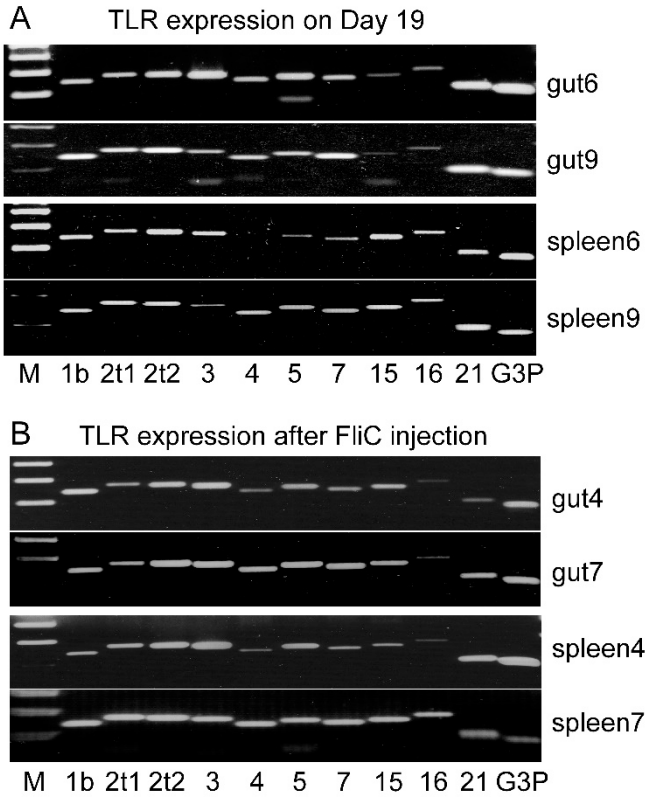

Figure 1. Expression profile of the chicken TLR repertoire. Reverse transcription polymerase chain reaction (RT-PCR) was performed on the indicated tissues of (A) non-injected and (B) FliC-injected embryonated eggs (Day-19) to determine TLR gene expression. PCR products were separated on 2% agarose gels and visualized with ethidium bromide. For each tissues, representative results from two chicken embryos are shown. Numbers on the horizontal axis represent the TLR class. M indicates 100, 200 and 300 base pair DNA markers. G3P: GAPDH.

Expression and Purification of Recombinant Salmonella *FliC*

As chTLR5 was found to be expressed at the late embryonic stage, we choose the FliC flagellin protein of *Salmonella* Enteritidis to test the immune response after *in ovo* immunization with purified protein. The FliC protein carries intrinsic chTLR5 stimulating (adjuvant) activity (29). The recombinant protein, which was cloned with *N*-terminal His-tag to enable purification, migrated on SDS-PAGE as a single protein band with the expected apparent molecular mass of 64 kDa. The protein band reacted with anti-FliC antibodies in Western blots (Fig. 2A). As the protein tended to aggregate in saline buffer, stock solutions were dissolved in 4 M urea. TLR activation assays using chTLR5

transfected HeLa 57A cells that carry an NF-κB luciferase reporter gene demonstrated that the purified FliC protein stimulated NF-κB activity in a chTLR5 dependent fashion (Figure 2B), confirming its intrinsic TLR5 stimulating activity (29).

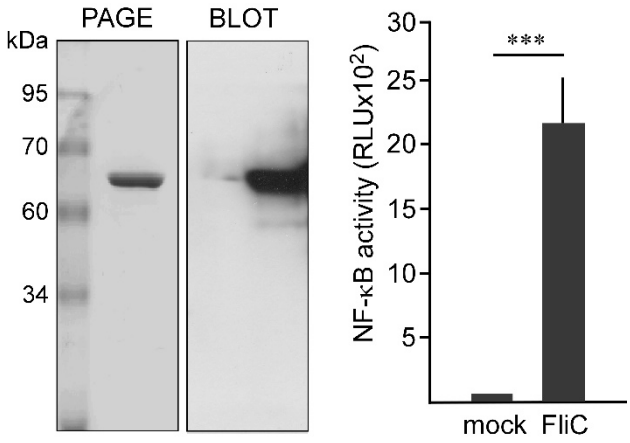

Figure 2. Purity and function of recombinant *Salmonella* FliC. (A) SDS-PAGE and Western blot of His-tagged *Salmonella* FliC antigen used for immunization. Protein was visualized with Coomassie Brillant Blue (gel) and FliC-specific antisera (blot). (B) NF-κB activation of HeLa-57A cells transfected with chTLR5 after exposure (5 h) to purified FliC or solvent. Stimulation of NF-κB is expressed as relative light units (RLU). Data are the mean ± SEM of three experiments. Molecular mass is indcated in kilodaltons (kDa).The asterisk indicates statistical significance (**P < 0.01).

Next, we injected 20 μg of the purified FliC protein into 18-day old embryonated eggs. RT-PCR analysis on RNA isolated from the gut and spleen tissue of the embryos isolated at Day-1 post-injection showed grossly similar TLR expression profiles as observed for the non-injected emmbryonated eggs with exception of chTLR15 (Figure 1B). The expression of this gene was much stronger in the gut tissue after the injection of FliC. ChTLR15 is activated by microbial proteases (39). ChTLR16 (TLR1a) remained weakly expressed in the gut tissue after flagellin exposure. Injection of FliC into the eggs did not influence the subsequent hatching and survival of the chicken.

Effect of FliC Injection on Cytokine Gene Expression

We also tested for changes in the expression of inflammatory genes in the gut and spleen tissue after the *in ovo* delivery of FliC protein. Quantitative RT-PCR transcript analysis on RNA isolated at Day-1 post-injection revealed that FliC induced a strong upregulation (30-75 fold) of chIL-6 and chIL-8 (CXCL8-CXCLi2) mRNA levels in both the small intestine and caecal tissue when compared to the levels present in the mock-injected embryos (Figure 3). The upregulation was not observed when the same volume of buffer was injected into the eggs. The injection of the flagellin did not induce significant changes in transcript levels of the genes in spleen tissue (Figure 3). Additional comparative transcript analysis demonstrated minimal differences in chIL-1β and chINF-β transcripts between the FliC-injected and mock-injected embryonated eggs for all tested tissues (Figure 3). Overall, the results indicate that *in ovo* delivery of FliC protein to 18-day chicken embryos induces a potent and specific intestinal innate immune response.

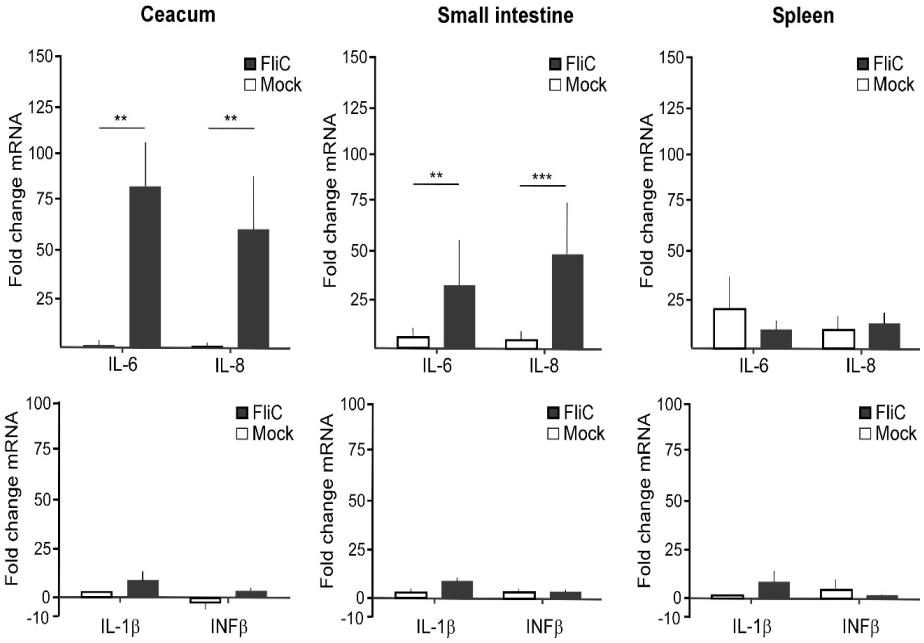

Figure 3. Expression of chIL-8 (CXCLi2), chIL-6, ch-IL1β, and chINFβ transcripts. Expression of the inflammatory genes in the indicated tissues at one day after *in ovo* delivery of purified FliC to 18 day-old embryonated eggs was determined by RT-qPCR. Results for each of the indicated genes are expressed as the mean ± SEM fold difference between the average mRNA level in the indicated tissues of the FliC- and mock-injected embryonated eggs compared to non-injected controls (n=4-5). Significant differences in ΔmRNA values between the FliC- and mock-injected groups were analyzed using log transformed data as described in Materials and Methods and are indicated by asterisks. ***P < 0.005; **P < 0.01.

Antibody Response after In Ovo *Protein Delivery*

To investigate the effect of *in ovo* immunization with FliC protein on the generation of an antibody response, the presence of FliC-directed IgY antibodies was followed in time. Sera were collected at Day-11 and Day-21 post-hatch from the FliC-injected, mock-injected and non-treated experimental groups of eggs. In the first experiment, embryonated eggs from SPF chicken were used to exclude an effect of possible maternal FliC-specific antibodies. The presence of anti-FliC antibodies was detected by enzyme-linked immunosorbent assay (ELISA) using purified FliC as antigen. Mean antibody titers were defined as the highest sera dilution giving statistically significant differences between the FliC-injected and mock-injected groups. Results showed a mean antibody titer of 1:1280 at Day-11 post-hatch for the FliC-injected group (Figure 4A). At Day-21 post-hatch, FliC antibody levels had

dropped to a mean titer of 1:160 (Figure 4B). Repetition of the experiment with embryonated eggs from a commercial chicken flock yielded similar results including the decline in antibody titer in the period between Day-11 and Day-21 (Figure 4C and 4D). In this experiment, the mean titer for the FliC-injected group declined from 1:640 on Day-11 to 1:80 on Day-21. Overall, the results indicate that i*n ovo* delivery of purified flagellin with TLR5 stimulating activity induces a potent immune response that can easily be detected at Day-11 post-hatch but that has declined at Day-21 of age.

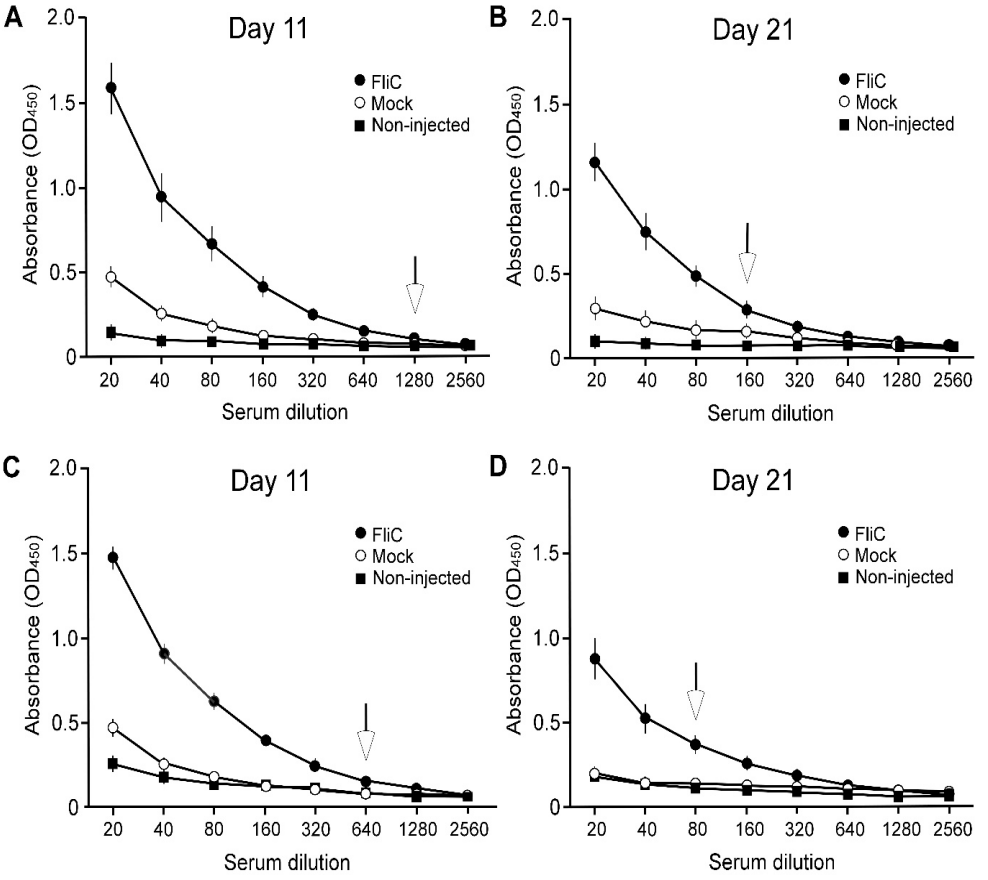

Figure 4. FliC-specific IgY reactivity in serially-diluted sera of FliC immunized embryos at Day-11 and Day-21 post-hatch as measured by ELISA. Both sera from hatched chicken from eggs of (A, B) SPF and (C, D) commercial flocks were tested. Sera from chicken hatched from mock-injected or non-injected embryonated eggs served as controls. The numbers on the X-axis represent the recipocral of the serum dilution. Data represent mean ± SEM values of the reactivity of individual chicken sera. The highest dilution which yielded a statistically significant difference in antibody reactivity between the FliC- and mock-injected groups is indicated with arrows.

DISCUSSION

The present study was designed to evaluate the ability of chicken embryos to mount an immune response after *in ovo* delivery of *Salmonella* flagellin. Evidence is provided that antigen injected into the amniotic fluid of 18-day-old fertilized eggs reaches the intestine of the embryo, evokes a local cytokine response, and gives rise to the development of flagellin-specific antibodies that can be detected until at least Day-21 post-hatch. The results indicate that at the late embryonic stage the chicken immune system is sufficiently mature to respond to *in ovo* injected protein antigens. This implies that *in ovo* immunization with protein-based subunit vaccines may be a feasible approach to limit bacterial infections early after hatching.

The strategy of *in ovo* vaccination as a method to protect young birds against infections is well established. The procedure is successfully used worldwide to deliver live attenuated vaccines that limit the spread of viral diseases such as Marek's disease and Newcastle disease (4, 41, 6, 26). To our knowledge, *in ovo* vaccination with protein subunit vaccines is much less developed and not commercially applied. We investigated the feasibility of this approach by systematic analysis of the antigen route, the evoked local intestinal innate response, and the antigen-specific antibody kinetics. We used *Salmonella* flagellin as an antigen as this protein is highly immunogenic in adult chickens. This immunogenicity may be partly attributed to the intrinsic activity of *Salmonella* flagellin to activate chTLR-5 (29, 42, 28, 43). Our results indicate that *in ovo* immunization with recombinant flagellin results in a potent adaptive immune response. This finding seems at variance with the reported incomplete development of the secondary immune organs in late embryonic and neonatal chicken (44). However, our results are in line with the response observed after *in ovo* delivery of non-replicating adenovirus-vector based vaccines (7, 8), heat-killed *Campylobacter* organisms (45) and recombinant *Eimeria* proteins (9, 11). These results strongly suggest that the chicken immune system at the late embryonic stage is sufficiently mature to respond to subunit vaccine antigens.

Both the intestinal accumulation of injected materials (46, 47) and the increased cytokine transcript levels in the intestinal tissue at 24 h after *in ovo* antigen delivery indicate that the delivered materials enter the intestine of the embryo via the oral route. The induction of mucosal chIL-6 and chIL-8 (CXCL8-CXCLi2) transcripts indicates that naive

embryonic intestinal tissue (i.e., not previously exposed to microbiota) is capable to respond to the delivered antigen. This effect is likely mediated through TLR5 as activation of recombinant chTLR5 by *Salmonella* FliC promotes chIL-6 and chIL-8 gene transcription (29). Additionally, our RT-PCR results clearly demonstrate intestinal chTLR5 expression at Day-19 of embryonic development. Previous analysis of TLR expression during chicken embryogenesis indicates the presence of transcripts as early as Day-3 of embryonic development (34) although expression patterns vary during subsequent embryonic development (33, 34). Notably, *in ovo* delivery of flagellin protein at Day-18 did not increase chIL-6 or chIL-8 transcript levels in spleen tissue, despite the presence of chTLR5. This suggests that the injected flagellin may not have been transported beyond the intestinal delivery site. Alternatively, it can be argued that the reported immature status of spleen immune tissue of the embryo (43) prevented an appropriate response.

The strong humoral immune response towards bacterial flagellin measured at Day-11 post-hatch clearly demonstrates that *in ovo* immunization of chicken embryos with purified protein also evokes a potent adaptive immune response. This result implies that not only the innate immune system but also the adaptive immune system is sufficiently mature at the late embryonic stage to respond to a single injection with purified antigen. This is important as the strategy of protein subunit immunization deviates from the successful *in ovo* immunization with live attenuated vaccines. After delivery of these vaccines, the virus may still replicate *in vivo* resulting in enhanced and prolonged exposure to the antigen. The recent use of bacterial flagellin as a constituent in viral vaccine (23) underlines the potency of the adjuvant activity of flagellin in the generation of a protective immune response.

A potential disadvantage of *in ovo* immunization with isolated protein antigens without additional boosting early post-hatch may be the rapid waning of antibody titers. Indeed, analysis of the sera of chicken collected at Days 11 and 21 post-hatch revealed persistent but gradually declining flagellin antibody titers. Yet, flagellin-specific antibodies could be detected for up to at least three weeks post-hatch, indicating that *in ovo* injection results a relatively long lasting humoral response. The observed gradual decline in anti-flagellin titer may reflect a reduced antibody production but may also be attributed to a dilution of the antibodies due to the rapid growth of the broiler chicken. If needed, boosting of the chicken with isolated flagellin (e.g. via drinking water) or by natural antigen exposure during the first weeks of life may reverse the waning of antibody levels (45). Of note, successful and commercially applied live attenuated vaccines against viral diseases

are considered to protect broilers for the duration of 4-6 weeks, when desired, longer protection can be achieved with re-vaccination.

Overall, our results indicate that *in ovo* immunization with recombinant bacterial flagellin protein evokes a potent humoral immune response that can be detected for up to at least three weeks post-hatch. This finding combined with those obtained for non-replicating virus vaccines and *Eimeria* proteins indicate that *in ovo* application of bacterial subunit vaccines is a feasible approach. Major target organisms of bacterial *in ovo* vaccines may be the pathogenic *E. coli* species and the dominant zoonotic pathogens *Salmonella* Enteritidis and *Campylobacter jejuni*. *In ovo* vaccination is attractive because of the low vaccine dose and the proven history of being safe and suitable for mass vaccination (48). A major challenge remains to identify conserved antigens that together with flagellin, provide sufficient cross-protection between strains.

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Chapter 3

Invasive behavior of *Campylobacter jejuni* in immunosuppressed chicken

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ABSTRACT

***Campylobacter jejuni* is a predominant cause of gastroenteritis in humans but rather harmless in chickens. The basis of this difference is unknown. We investigated the effect of the chicken immune defense on the behavior of *C. jejuni* using glucocorticoid (GC)-treated and mock-treated 17-day old Ross 308 chicken bearing in mind that GCs have immunosuppressive effects and dampen the innate immune response. The effect of GC administration on the behavior of *C. jejuni* was compared with that on infection with *Salmonella* Enteritidis to address possible microbe-associated differences. Our results revealed that GC treatment fastened the intestinal colonization of *C. jejuni* ($p<0.001$) and enhanced its dissemination to the liver ($p=0.007$). The effect of GC on intestinal colonization of *S.* Enteritidis was less pronounced ($p=0.033$) but GC did speed up the spread of this pathogen to the liver ($p<0.001$). Cytokine transcript analysis showed an up to 30-fold reduction in baseline levels of *IL-8* mRNA in the cecal (but not spleen) tissue at Day 1 after GC treatment ($p<0.005$). Challenge with *C. jejuni* strongly increased intestinal *IL-8*, *IL-6*, and *iNOS* transcript levels in the non-GC treated animals but not in the GC-treated birds ($P<0.005$). *In vitro* assays with chicken macrophages showed that GC dampened the TLR agonist- and *C. jejuni* induced-inflammatory gene transcription and production of nitric oxide ($P<0.005$). Together, the results support the hypothesis that *C. jejuni* has the intrinsic ability to invade chicken tissue and that an effective innate immune response may limit its invasive behavior.**

INTRODUCTION

The bacterial food-borne pathogen *Campylobacter jejuni* (*C. jejuni*) is estimated to cause about 100 million cases of diarrheal illness each year (1,2). One major source of *C. jejuni* is contaminated chicken meat. *C. jejuni* frequently colonizes the ceca of chicken at high concentrations (10^9 CFU/g cecal content) usually without or with very mild clinical manifestations (3-7).The molecular basis of the different effects of *C. jejuni* in humans and chickens is still unknown. Factors that may contribute include a differential expression of bacterial virulence traits in the different hosts, a variable composition of the local microbiota, intrinsic differences in the intestinal mucosal architecture, and/or differences in immune defense between the species. Detailed analysis of the interaction of *C. jejuni* with chicken cecal tissue has yielded variable results ranging from a lack of *C. jejuni* penetration of the intestinal mucus layer to *C. jejuni* invasion of chicken tissue with signs of a local inflammatory response (6-10).

One important step in the mucosal defense against bacterial pathogens is the early recognition of microbial products by the innate immune system. One class of host receptors that signals the presence of bacteria is the Toll-like receptor (TLR) family. Activation of members of this receptor family by bacterial ligands initiates downstream signaling events that result in nuclear translocation of transcription factors (such as NF-ƙB) that regulate the expression of pro- or anti-inflammatory genes. Comparative analysis of the human and chicken TLR repertoire revealed several differences between the mammalian and avian species (for a review see refs. 11, 12). Chicken-specific TLR characteristics include a broader subset of TLR1/2 receptors (13), the absence of a TLR4/MyD88-independent signaling pathway (14), the presence of a protease-activated TLR15 (15), and the presence of TLR21 as a functional orthologue of human TLR9 (16, 17). *C. jejuni* can activate both human and chicken TLRs (18) but the contribution of TLRs to the chicken defense against *C. jejuni* remains to be determined.

One major class of regulators of mammalian biological systems including the immune system is the adrenal-derived glucocorticoids. These steroid hormones bind to the cytosolic glucocorticoid (GC) receptor. The formed complex translocates to the nucleus and binds to response elements in the promoter regions of GC-responsive genes (19, 20). GCs also influence transcription factors that induce potent anti-inflammatory activity (19, 21, 22).

GCs are always detectable in serum, but their concentration strongly varies with the environmental stress encountered by the host. The hypothalamus-pituitary-adrenal axis responsible for the stress-related production of GC in mammals is also functional in chicken (23, 24). On poultry farms, environmental stress influences serum corticosterone levels and affects the susceptibility of chicken to infectious diseases (25, 26), possibly through its dampening effect on the immune system.

In the present study, we applied GC-induced immunosuppression to investigate the contribution of the chicken immune defense to the apparent commensal behavior of *C. jejuni* in chicken. GC-treated and control chickens were challenged with *C. jejuni* or (as control) with *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis). Bacterial colonization, systemic dissemination, and tissue expression of pro-inflammatory genes were followed in time. In addition, the effect of GCs on *C. jejuni*-induced chicken innate immune (TLR) activation was assessed in cultured chicken cells. Our results demonstrate that bacterial challenge of GC treated chicken results in a poor inflammatory response and a more rapid intestinal colonization and dissemination of *C. jejuni.*

RESULTS

Effect of glucocorticoids on the colonization and dissemination of C. jejuni in chicken

To investigate the reason for the behavior of *C. jejuni* in chicken, we first investigated the effect of GC administration on the intestinal colonization and dissemination of *C. jejuni*. Hereto, different groups of SPF chicken were injected with either the glucocorticoid Depo-Medrol (groups 1 and 3) or PBS (groups 2 and 4). After 24 h, groups 3 and 4 were challenged orally with 10^5 CFU of *C. jejuni* strain 81116. Groups 1 and 2 served as non-challenged controls. The following days, at least five chicken of each group were sacrificed to determine the number of *C. jejuni* in the ceca and liver, and to isolate tissue RNA for gene expression analysis.

Enumeration of *C. jejuni* in the collected cecal contents demonstrated significantly higher loads of *Campylobacter* in the ceca of the GC-treated group (group 3) compared to those of the PBS-injected group (group 4) (β=0.56, 95%CI 0.26-0.86, $p<0.001$) (Fig. 1A, left panel). In the GC-treated animals, the number of *C. jejuni* had geometric mean values of $3.0x10^9$ CFU/g content at Day 2 to $4.5x10^9$ CFU/g content at Day 4 after challenge. In the control animals, *C. jejuni* values reached mean levels of $1.0x10^3$ and $1.5x10^6$ CFU/g at

Days 2 and 3 respectively, rising to $7.1x10^7$ CFU at Day 4. Bacterial culture of liver tissue demonstrated significantly higher numbers of *C. jejuni* in the liver of the GC-treated group compared to the control group (β=0.46, 95%CI 0.13-0.79, p=0.007). At Day 2 and Day 3 after challenge, the number of *C. jejuni* in the liver reached mean values of $2.2x10^3$ CFU/g and $4.1x10^3$ CFU/g, respectively. At these time points, *C. jejuni* was still virtually absent in the liver of the control animals (1.1 CFU/g tissue at Day 2 and 16.7 CFU/g tissue at Day 3) (Fig. 1A, right panel).

Experiments with chicken challenged with *S.* Enteritidis strain CVI-1 instead of *C. jejuni* revealed a slightly faster intestinal colonization (β=0.10, 95%CI 0.01-0.19, p=0.033) (Fig. 1B, left panel) and much faster bacterial spread to the liver (β=0.75, 95%CI 0.47-1.02, p<0.001) (Fig. 1B, right panel) for the GC-treated group (group 5) compared to the control group (group 6).

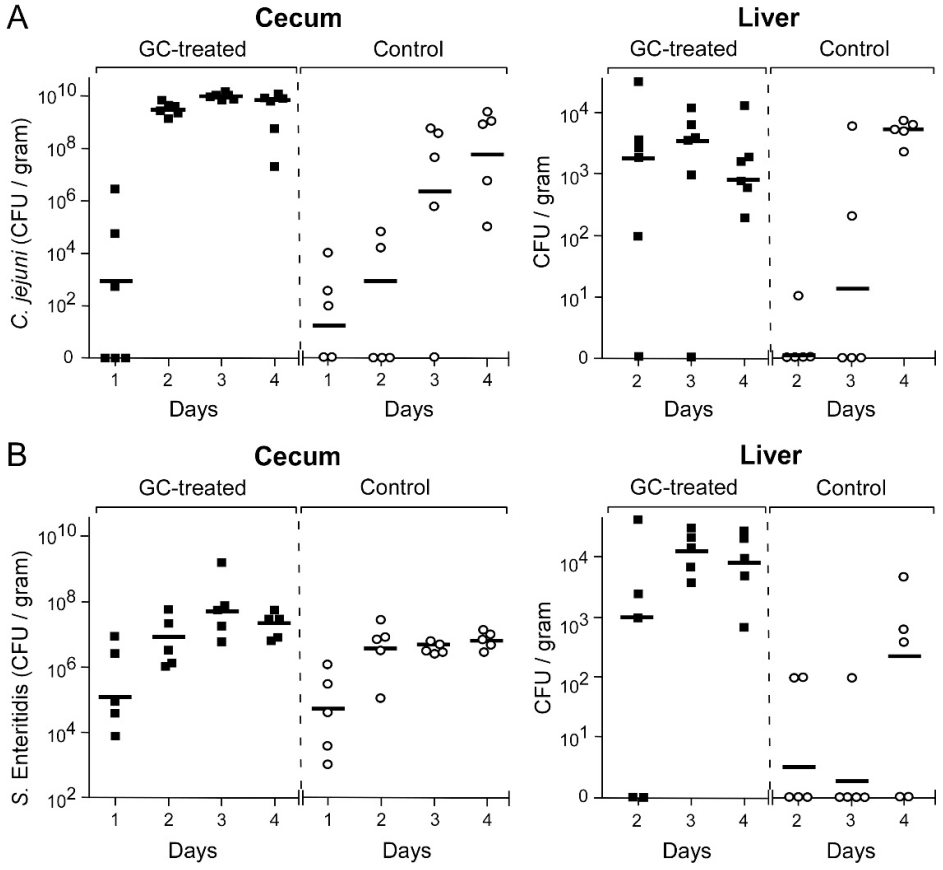


Figure 1. *C. jejuni* and *S.* Enteritidis colonization kinetics in GC-treated and control chickens. Chicken were injected with GC (closed blocks) or PBS (open circles) and 24 h later challenged orally with 10^5 CFU of *C. jejuni* or *S.* Enteritidis. At Day 1-4 post-challenge, *C. jejuni* (panel A) and *S.* Enteritidis (panel B) colonization of the ceca and the liver was estimated by CFU counting. Data are plotted as CFU per gram of cecal content or liver tissue for each chicken and expressed as the geometric mean (horizontal bars) of CFU per group of chicken. Statistical analysis of differences between treated and non-treated chickens were calculated with a gamma generalized linear model and gave the following values: *Campylobacter* in the ceca: β=0.56, 95%CI 0.26-0.86, p<0.001' *Campylobacter* in the liver: β=0.46, 95%CI 0.13-0.79, p=0.007; *Salmonella* in the ceca: β=0.10, 95%CI 0.01-0.19, p=0.033; *Salmonella* in the liver: β=0.75, 95%CI 0.47-1.02, p<0.001.

***Inflammatory gene expression in chicken challenged with* C. jejuni**

To learn more about the mechanism(s) contributing to the more rapid colonization and dissemination of *C. jejuni* after GC administration, we first determined the effect of the *C. jejuni* challenge on the expression of inflammatory genes for the non-GC treated animals. RT-qPCR analysis on mRNA derived from cecal tissue at Day 1 after challenge with *C. jejuni* (group 4) showed a 50-100 fold up-regulation of *IL-6*, *IL-8*, and inducible nitric oxide synthase (*iNOS*) transcripts when compared to transcript levels in the non-challenged control group (group 2) (Fig. 2A). At Day 4 after bacterial challenge, the difference in cecal *IL-6*, *IL*-8, *IL-1β* and *iNOS* transcripts was less pronounced (Fig. 2A). In spleen tissue, the challenge with *C. jejuni* caused an increase in *IL-6* and *IL-8* transcripts at Day 1 and an additional increase in *IL-1β* mRNA at Day 4. The bacterial challenge did not change *iNOS* and *IFNβ* transcript levels (Fig. 2B).

To determine the microbe specificity of the host response, we performed similar transcript analysis after challenge with *S.* Enteritidis. This pathogen induced an increase in cecal *IL-8* and *IL-6* transcript levels at Day-1 and Day-4. Transcript levels of iNOS did not differ between the infected and non-infected groups of animals (Fig. 2C). This result clearly differed from the cecal response upon challenge with *C. jejuni* (cf. Figs. 2C versus 2A). Like *C. jejuni*, infection with *S.* Enteritidis variably increased *IL-8*, *IL-6* and *IL-1β* transcript levels in spleen tissue (Fig. 2D). Neither *C. jejuni* nor *S.* Enteritidis significantly increased *iNOS* transcript levels in the spleen (Figs 2B and D). Overall, our results indicate that colonization of chicken with *C. jejuni* induces a robust host response and that *C. jejuni* and *S*. Enteritidis elicit bacteria- and tissue-specific cytokine responses.

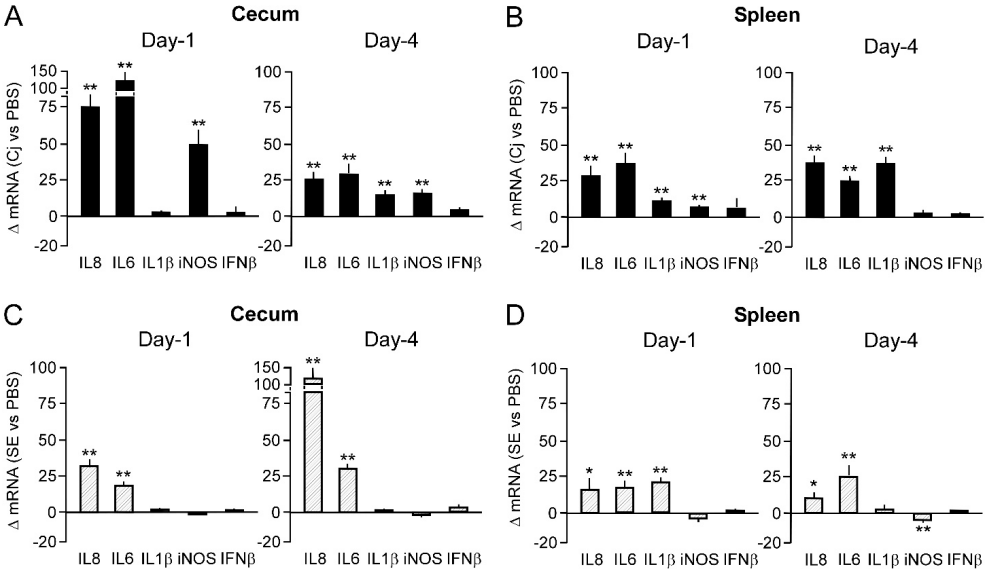


Figure 2. Effect of *C. jejuni* and *S.* Enteritidis colonization on cytokine mRNA levels. Transcript levels for the indicated cytokines were determined in cecal mucosa and spleen tissue isolated from individual birds at Day 1 and 4 after challenge with *C. jejuni* (panels A and B) or *S.* Enteritidis (panels C and D). RT-qPCR results were expressed as fold difference between the average mRNA levels in the indicated tissues of challenged chicken compared to (PBS-injected) control birds. Significant differences in ΔmRNA values were analyzed using log transformed data as described in Materials and Methods. Significant differences are indicated: **P<0.005; *P<0.05.

Glucocorticoid-induced downregulation of pro-inflammatory gene expression

Transcript analysis on the same panel of inflammatory genes at Day 1 after administration of GC (i.e. without additional bacterial challenge) (group 1) revealed a 30-fold reduction of cecal baseline *IL-8* mRNA levels compared to those in the PBS-injected control group (Fig. 3). At Day 4, only a minor difference in the cytokine mRNA levels between both groups was measured (Fig. 3). RT-qPCR assays on spleen tissue revealed no significant differences in baseline transcripts between the GC-treated and control group during the period of analysis (Fig. 3). The transient downregulation of baseline cytokine transcript levels in the ceca may reflect the suppression of the mucosal immune response elicited by the commensal bacterial flora. Notably, comparison of cytokine transcript levels between PBS-

injected and non-injected chickens revealed no differences (data not shown), indicating that the acute stress that may be associated with the handling of the animals did not cause changes in the expression of the tested genes.

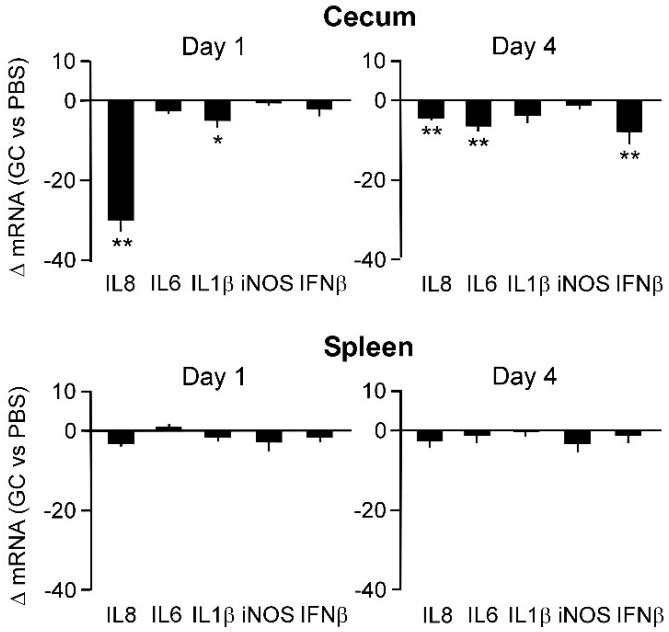


Figure 3. Effect of GC treatment on inflammatory gene expression in cecal mucosa and spleen tissue. Transcript levels of the indicated genes at Day 1 and 4 after injection of chicken with GC or PBS were determined by real-time RT-qPCR. Results are expressed as the mean ± SEM fold difference in tissue mRNA levels in the GC-treated versus control animals. Significant differences in ΔmRNA values were analyzed using log transformed data as described in Materials and Methods. Significant differences are indicated: **P<0.005; *P<0.05.

Effect of glucocorticoids on C. jejuni *induced inflammatory gene transcripts*

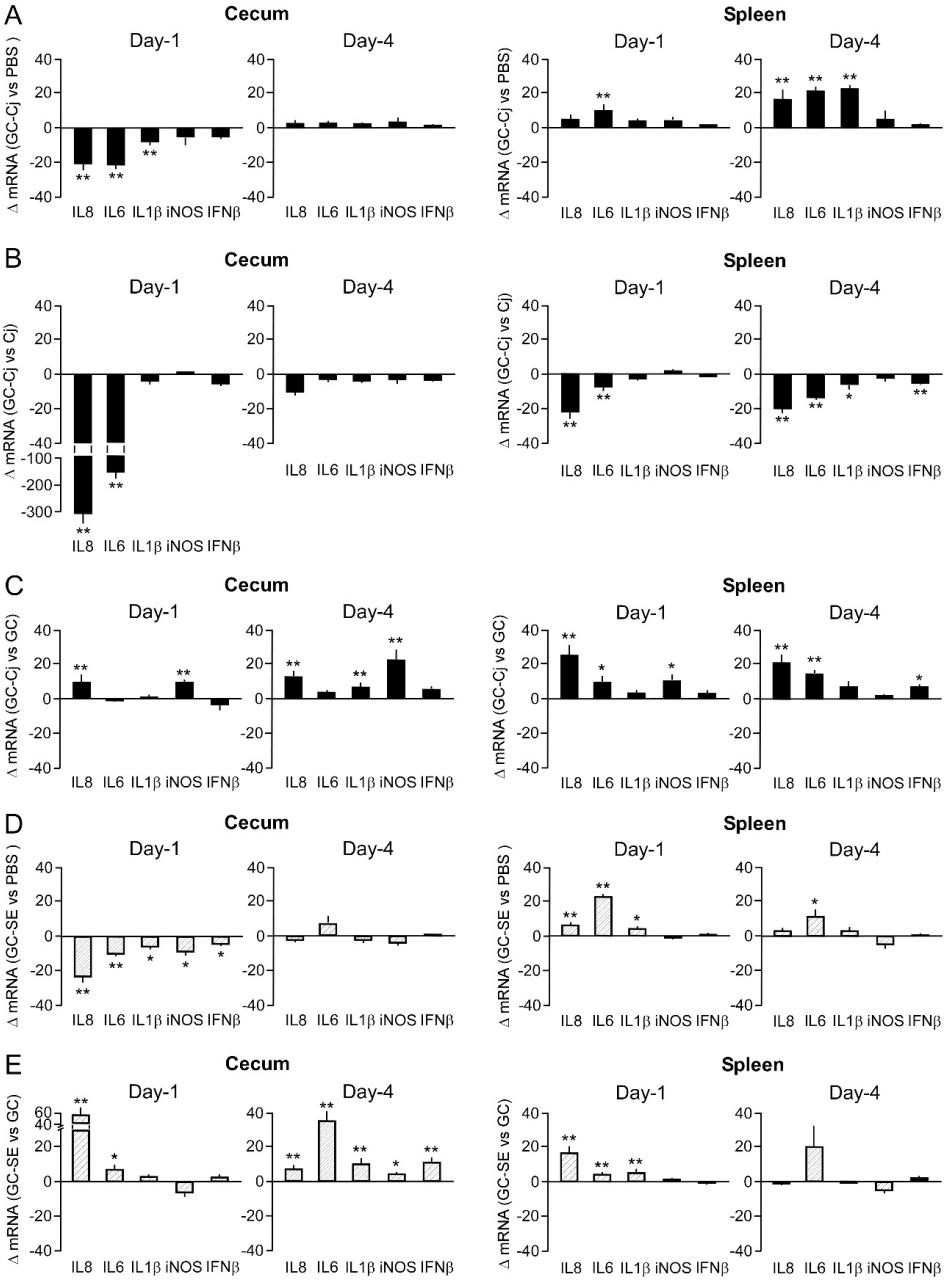
Next, we determined cytokine transcript levels for the complex combination of GC treatment plus bacterial challenge. Comparison of transcript levels in tissues from the GC-treated, *C. jejuni* challenged birds (group 3) with those in the PBS-injected, non-challenged animals (group 2) demonstrated significantly reduced cecal mRNA levels for *IL-6*, *IL-8*, and *IL-1β* at Day-1 post challenge. In spleen tissue, the effect was limited to a minor increase in *IL-6* mRNA in the GC-treated, *C. jejuni* challenged group (Fig. 4A). At Day 4 post-challenge, the reduced cytokine transcript levels in cecal tissue of the GC-treated and challenged animals had returned to baseline levels. At this point in time, a moderate

increase in multiple pro-inflammatory gene transcripts was measured in spleen tissue (Fig. 4A).

To more specifically determine the strong dampening effect of the GC treatment on the *C. jejuni*-induced tissue response, we compared gene transcript levels in the GC-treated, *C. jejuni* challenged animals (group 3) with those in the non-GC treated, *C. jejuni*-challenged chickens (group 4). This demonstrated that GC treatment reduced the *C. jejuni*-induced *IL-8* and *IL-6* response by >150-fold at Day 1 post-challenge (Fig. 4B). On Day 4, the strong immunosuppressive effect had virtually disappeared. In spleen tissue, GC treatment reduced several of the measured *C. jejuni*-induced cytokine transcripts by a factor 5 to 20 on both Day 1 and Day 4 (Fig. 4B).

To learn whether *C. jejuni* was still able to induce a cytokine response in the GC-treated birds, we compared the gene transcript levels in the cecal tissue of the GC-treated, *C. jejuni* challenged animals (group 3) with those of the GC-treated, non-challenged chickens (group 1). This showed only a small increase in the cecal *IL-8* and *iNOS* mRNA levels after *C. jejuni* challenge (Fig. 4C). This indicates that GC treatment largely prevents the robust *C. jejuni*-induced inflammatory response that is observed after *C. jejuni* infection of non GC-treated chickens (Fig. 2A). This effect may contribute to the observed more rapid colonization of the ceca and liver in these animals (Fig. 1).

Figure 4. Effect of the combination of GC treatment plus challenge with *C. jejuni* or *S.* Enteritidis on inflammatory gene expression. Chicken were injected with GC or PBS and after 24 h challenged with *C. jejuni* (panels A and C) or *S.* Enteritidis (panels D and E). Real-time RT-qPCR was performed on mRNA isolated from cecal mucosa and spleen tissue collected at Days 1 and 4 post-challenge. Results are expressed as the mean ± SEM fold difference in mRNA levels in GC-treated, treated and challenged chicken versus PBS-injected and non-challenged animals (panels A and D), PBS-injected and *C. jejuni* challenged chicken (panel B), or GC-treated and non-challenged chicken (panels C and E). Significant differences in ΔmRNA values were analyzed using log transformed data as described in Materials and Methods. Significant differences are indicated: **P < 0.005; *P < 0.05.


A
Cecum
Spleen
Day-1
Day-4
Δ mRNA (GC-Cj vs PBS)
Δ mRNA (GC-Cj vs PBS)
B
Δ mRNA (GC-Cj vs Cj)
C
Δ mRNA (GC-Cj vs GC)
D
Δ mRNA (GC-SE vs PBS)
E
Δ mRNA (GC-SE vs GC)
IL8 IL6 IL1β iNOS IFNβ

Effect of glucocorticoids on* S. *Enteritidis induced inflammatory gene transcripts

Comparative analysis of gene transcripts in tissues derived from GC-treated chicken challenged with *S.* Enteritidis (group 5) with those from non-infected control chickens (group 2) indicated that the GC treatment inhibited the *Salmonella*-induced increase in intestinal *IL-8* and *IL-6* response at both Day-1 and Day-4 (cf. Fig. 4D vs 2C). In spleen tissue the changes in *IL-8*, *IL-6* and *IL-1β* transcript levels were also less pronounced than in the non-GC treated, *S.* Enteritidis challenged animals (cf. Fig. 4D vs 2D). When the transcript data of the GC-treated, non-challenged animals were used as a reference, moderate increases in mainly *IL-8* (Day-1) and *IL-6* transcript levels (Day-4) were measured for both intestinal and spleen tissue samples (Fig. 4E). Overall, these data point to the presence of a GC treatment-induced attenuation of the host response towards *S.* Enteritidis, as was seen for *C. jejuni*.

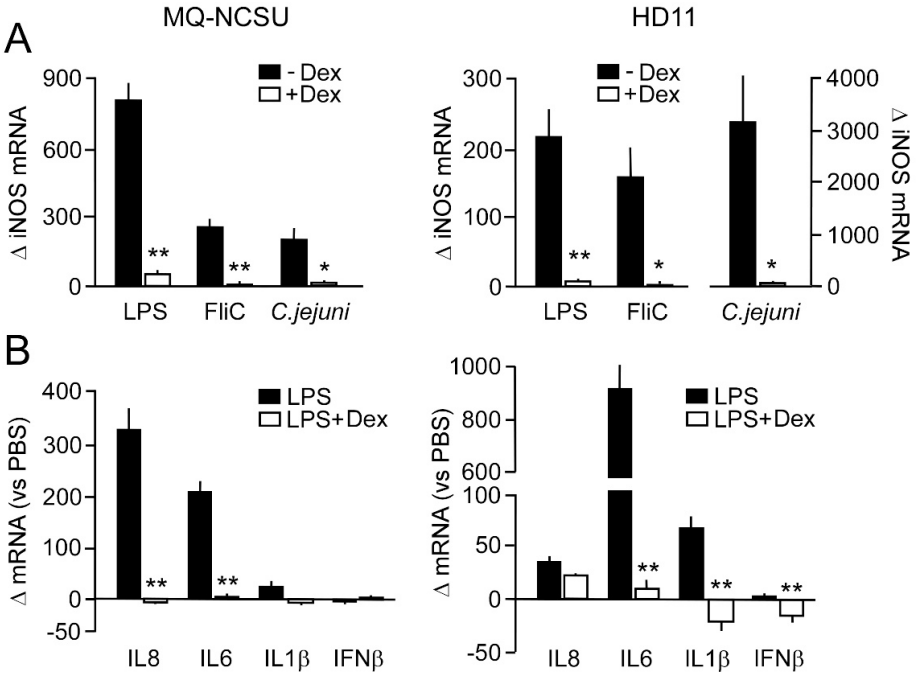


Figure 5. Effect of GC-treatment gene TLR- and *C. jejuni*-induced transcription in chicken macrophages. (A) MQ-NCSU and HD11 cells pre-incubated (17 h) with or without dexamethasone were stimulated with LPS (100 ng/ml) or flagellin (FliC, 1 µg/ml) for 16 h or with live *C. jejuni* ($2x10^5$) for 8 h. Then, real-time RT-qPCR was performed on mRNA isolated from the cells to measure differences in iNOS mRNA levels between the Dex-treated and control cells. (B) Fold difference in the indicated inflammatory gene transcript levels in the GC-treated and non-GC treated cells after stimulation with LPS (100 ng/ml, 16 h). All results are expressed as the mean ± SEM fold difference in the transcript levels between the Dex-treated ands control cells (n=3). For each response significant differences between Dex-treated and control cells are indicated: **P<0.001; *P<0.05.

GC modulation of the TLR response

In search for a potential explanation of the apparent attenuated inflammatory response in the GC-treated chickens, we investigated the expression of inflammatory genes and the production of nitric oxide (NO) in two different chicken macrophages cell lines (MQ-NCSU and HD11). Hereto, cells were exposed to the glucocorticoid dexamethasone (10^{-6} M) or solvent solution for 17 h prior to the addition of defined bacterial TLR agonists. RT-qPCR analysis on mRNA isolated from the non GC-treated macrophages demonstrated a strong induction (150-200 fold) of *iNOS* transcript after exposure of the cells to LPS, flagellin, and live *C. jejuni* strain 81116 (Fig. 5A). GC pretreatment of the cells fully

abrogated this response in both cell lines. A similar strong reduction was measured for the *IL-8* and *IL-6* response in MQ-NCSU cells and for the *IL-6*, *IL-1β* and *IFNβ* transcripts in HD11 cells (Fig. 5B).

To ensure that the observed changes in inflammatory gene transcription translated to the protein level, we measured the production of cellular nitric oxide (NO) under the different conditions. This revealed that both bacterial LPS and flagellin (FliC) induced a dose-dependent increase in NO production in the chicken macrophages (Fig. 6A and B, filled bars), in line with the observed induction of *iNOS* mRNA (Fig. 5A). Again, GC pretreatment (10^{-6} M, 17 h) of the cells strongly reduced these responses (Figs. 6A and B, open bars). The inhibitory effect required preincubation of the cells with GC. The effect was not observed when GC and the TLR agonists were added simultaneously (data not shown). Together, these results indicate that GC strongly inhibits both the TLR agonist- and bacteria-induced stimulation of the inflammatory response in chicken macrophages. This is in line with the observed attenuated *in vivo* response and may contribute to the observed invasiveness of *C. jejuni* in GC-treated chickens.

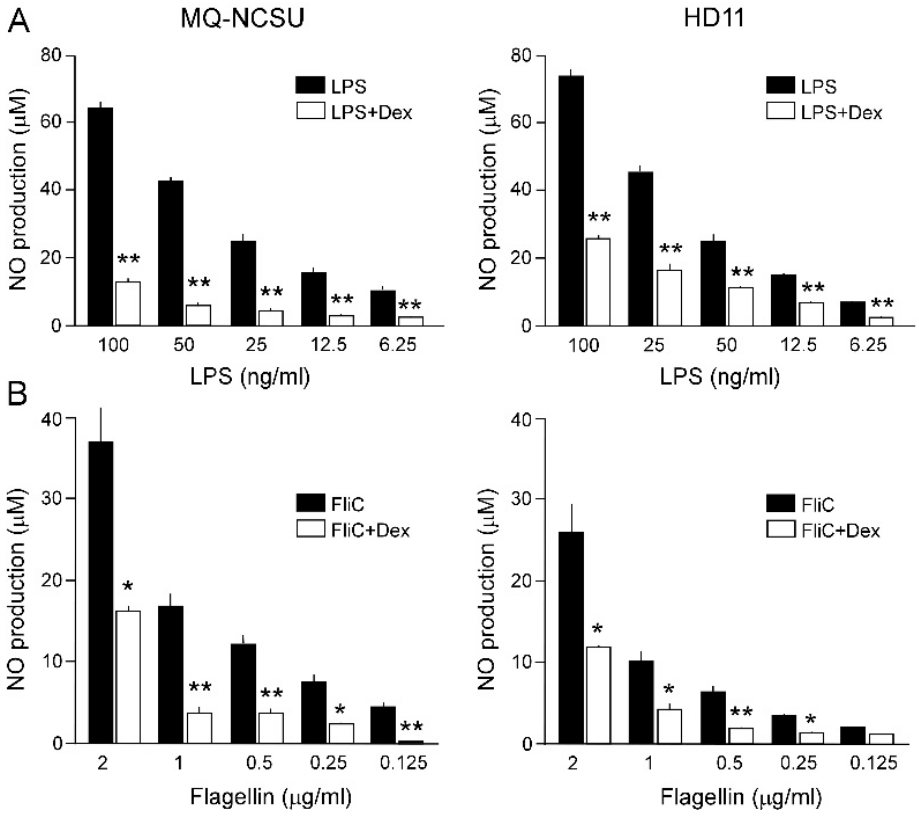


Figure 6. Effect of GC treatment on LPS or flagellin-induced production of nitric oxide in chicken macrophages. NCSU and HD11 chicken macrophages were incubated with dexamethasone (Dex, 10^{-6} M) for 17 h and then stimulated with the indicated concentrations of LPS (A) or flagellin (FliC) (B). After 24 h of stimulation, NO production was measured using the Griess assay. Results are the mean ± SEM of six (A) and four (B) experiments. Significant differences in ligand-induced NO production between Dex-treated and control cells are indicated: **P<0.005; *P<0.05.

DISCUSSION

In the present study, we investigated the role of the host immune defense in the behavior of *C. jejuni* in chickens. We provide evidence that GC treatment results in more rapid intestinal colonization and dissemination of *C. jejuni* to the liver in conjunction with a reduced pro-inflammatory gene expression at the infection niche. Stimulation of macrophages with different TLR ligands demonstrated strong inhibition of the chicken TLR

response for GC-treated cells confirming that GC weakens at least one component of the innate host defense. Our results support the concept that *C. jejuni* has the intrinsic property to spread in chicken but that the natural host defense may limit *C. jejuni* invasion and dissemination to distant organs.

The rationale for our study was the still unexplained host specificity of *C. jejuni* infection. *C. jejuni* is the leading cause of bacterial enterocolitis worldwide but relatively rarely induces clinical manifestations in the chicken (8, 10). Potential reasons for this difference are numerous and may range from a different expression of bacterial virulence traits or mucosal receptors to the existence of a different microbiota in the human and chicken host. Alternatively, it can be imagined that chickens have a more effective host defense against *C. jejuni*, although this appears to vary between chicken breeds (7, 27). We investigated the influence of the chicken immune response on the colonization and dissemination of *C. jejuni* in chicken by administering GC to the animals prior to *C. jejuni* challenge. GC has immunosuppressive effects, also in the chicken (28, 29). Our results indicate that administration of GC to chicken indeed results in a downregulation of baseline transcript levels of distinct inflammatory genes in the cecal tissue (Fig. 3). This result likely reflects the GC-induced dampening of the innate immune response.

Challenge of Ross 308 chicken with *C. jejuni* strain 81116 resulted in a more rapid colonization of the ceca and bacterial spread to the liver in the GC-treated animals (Fig. 1). The faster colonization of *C. jejuni* after GC treatment was unexpected. This effect was less pronounced for *S.* Enteritidis and has not been observed with *S.* Typhimurium (30). One possible explanation for the more rapid colonization with *C. jejuni* may be that GC treatment induces *C. jejuni* favorable alterations in the intestinal microenvironment by influencing e.g. the composition of the microbiota, the production or composition of the mucus, or the status of the local antimicrobial defense (6, 31). Alternatively, the lack of a potent innate immune response may contribute to the rapid bacterial expansion after GC treatment.

The rapid dissemination of *C. jejuni* to the liver in the GC-treated birds indicates that the bacterium has the intrinsic ability to spread to distant organs. Dissemination of *C. jejuni* to the blood and liver of chickens occurs infrequently and seems to vary between bacterial strains and chicken lines (5, 32, 33). Multiple mechanisms may contribute to the dissemination in the GC-treated animals. One hypothesis is that the more rapid cecal colonization (3.10^9 CFU at Day-2 after challenge) simply results in a more rapid bacterial

dissemination. Another is that GC treatment causes a general increased translocation of bacteria across the intestinal barrier. We considered these explanations as less likely as GC treatment has been reported to support rather than weaken intestinal barrier function(34, 35). We favored the scenario that the GC-induced dampening of the local innate immune response facilitates the bacterial spread to distant organs.

Analysis of the expression of inflammatory genes in the cecal tissue after challenge with *C. jejuni* revealed a strong increase *IL-6*, *IL-8* and *iNOS* transcripts. This pro-inflammatory signature in the non-GC treated animals indicates that *C. jejuni* is sensed by the chicken immune system and elicits a potent local immune response as previously noted (7-10, 36-38). The measured gene expression levels induced by *C. jejuni* were relatively high but this may obviously vary between chicken lines and depend on the composition of the microbiota which likely determines the baseline cytokine transcript levels. GC treatment reduced the baseline transcript levels in the cecal tissue and these levels barely increased after challenge with *C. jejuni* (Fig. 4B). This lack of response is likely caused by the immunosuppressive effect of GC on the mucosal cells but perhaps also by a reduced influx of inflammatory cells into the mucosal tissues. Both instances result in a weakened host defense and thus may contribute to the more invasive behavior of *C. jejuni*.

To ascertain that GC administration can limit the innate immune response in chicken cells, we tested the effect of GC on chicken macrophages *in vitro*. Avian cells respond to *C. jejuni* with increased transcript levels of cytokines and chemokines and the production of nitric oxide (18, 39-41). This response may involve the activation of different types of pathogen recognition receptors. One major molecular mechanism via which GC may limit the mucosal host defense is the inhibition of the TLR response (42, 43). TLRs are key sensors of environmental danger signals including microbial products and are major drivers of the innate immune response. The chicken TLR repertoire is well established and has been shown to respond to *C. jejuni (*18). Our finding that GC administration severely dampens chicken macrophage gene transcription and nitric oxide production in response to the TLR agonists LPS (TLR4) and flagellin (TLR5) as well as after exposure to *C. jejuni* (Figs. 5 and 6) shows that the TLR pathway in chickens is responsive to GC. These results support the scenario that GC administration contributes to the invasive behavior of *C. jejuni* at least partially by suppression of the local innate immune response. To some extent, this

situation may resemble observations in mice that show that animals with innate immune deficiencies become prone to *C. jejuni* infection (44-46).

Overall, our results for the first time demonstrate that GC treatment of chickens dampens the intestinal immune defense and causes a more rapid colonization and dissemination of *C. jejuni* in chicken. The data imply that *C. jejuni* has the intrinsic ability to invade chicken tissue and that the innate defense is important to limit this invasive behavior.

MATERIALS AND METHODS

Bacterial culture

C. jejuni strain 81116 (NTCT 11828)(47,48) was grown on Blood agar base II medium (Oxoid) containing 5% horse blood lysed with 0.5% saponin at 42^{o}C under microaerophilic (5% O_2, 10% CO_2, 85% N_2) conditions. S*almonella enterica* serovar Enteritidis (*S.* Enteritidis, nalidixic acid resistant) strain CVI-1 was grown on Luria-Bertani (LB) plates (Biotrading) at 37^{o}C. Bacteria from cloacal swabs and serial dilutions of tissue homogenates were grown on *Campylobacter* selective blood-free agar plates with CCDA-selective (Oxoid SR0155E) supplement (*C. jejuni*) or on Brilliant Green Agar supplemented with 100 µg/ml of nalidixic acid (*S.* Enteritidis) to suppress growth of resident flora. For use in challenge experiments, *C. jejuni* and S. Enteritidis were grown in Heart Infusion broth and Brain Heart Infusion broth (Biotrading) with nalidixic acid (Sigma-Aldrich) respectively, collected by centrifugation (4,000 x *g*, 10 min), and resuspended in PBS. Bacterial suspensions (10^5 CFU in 0.25 ml) were administered orally to the chicken using a 1 ml syringe that was carefully placed deeply into the mouth (7 cm).

Cell culture

The chicken macrophage HD11(49) and MQ-NCSU(50) cell lines were propagated in 25 cm^2 tissue culture flasks (Corning) containing 5 ml of Dulbecco's modified Eagle medium (Life Technologies) supplemented with 5% fetal bovine serum (FBS) at 37^{o}C and 10% CO_2. For use in cell stimulation assays, cells were seeded onto 12- or 24-well plates (10^5 cells/well) in 1 ml of DMEM plus 5% FBS per well.

Chicken experiments

Fertilized SPF (*Campylobacter* and *Salmonella* free) chicken eggs (Ross 308) were kept at 38^{o}C and 65-75% relative humidity in a forced air chicken egg incubator. After hatch, the chickens were divided into six groups (1-6) of 27 birds and housed in pens with *ad libitum* access to water and feed. Bacterial cultures of cloaca swabs taken at Day 1 after hatch confirmed the *Campylobacter* and *Salmonella* negative status of the animals. At Day 17 after hatch groups 1, 3, and 5 received the synthetic glucocorticoid methylprednisolone (Depo-Medrol®, intramuscular, 10 mg/kg body weight; Pfizer), while groups 2, 4, and 6 were injected with phosphate buffered saline (PBS). At 24 h after administration, groups 3 and 4 were challenged orally with 10^5 CFU of *C. jejuni* strain 81116, while group 5 and 6 were challenged with the equivalent number of *S.* Enteritidis. Groups 1 and 2 served as non-challenged controls.

At Days 1, 2, 3, and 4 after bacterial challenge, at least five birds from each group were sacrificed by cervical dislocation by professional staff to be able to determine the number of *C. jejuni* or *S.* Enteritidis in the cecal contents and liver, and to collect tissues for transcript analysis (see below). For bacterial enumeration, serial dilutions of 1 g of cecal content or 1 g of liver tissue homogenized in 3 ml of peptone water were grown as described above. The number of colonies (CFU) was counted after 24-48 h of incubation. Collected tissues were snap-frozen in liquid nitrogen and stored at -80^{o}C until further analysis. All chicken were cared for in accordance with accepted procedures of the Dutch law of animal welfare and all animal experiments were approved by the Ethics Committee of the Central Veterinary Institute of Wageningen University, Lelystad, the Netherlands.

A gamma generalized linear model with a log link function (as the CFU data were continuous, positive, right-skewed and with both constant variance and normally distributed residuals on the log scale) was used to indicate statistically significant differences between treated and non-treated chicks. This method allows assessing the overall effect of GC treatment on *Campylobacter* and *Salmonella* CFUs in the chicks' ceca and livers over the entire course of bacterial colonization, while accounting for the day of sampling.

***In vitro* treatment of cultured chicken cells**

To measure the effects of GC on cultured chicken cells, dexamethasone (Sigma) or the equivalent amount of solvent (ethanol, final concentration <0.5%) was added at 12 h after

seeding of the cells onto the 12- or 24-well plates (low cell density). Seventeen hours later, cells were stimulated with the indicated amounts of purified LPS, flagellin, or bacteria. After the indicated incubation periods (37^{o}C, 10% CO_2), cell culture supernatants were collected for measurement of nitric oxide (NO). The cells were treated with RNA-Bee^{TM} (Bio-connect) to extract RNA for transcript analysis (see below). Purified LPS and *Salmonella* flagellin (FliC) were isolated as previously described (14, 51).

RNA isolation from tissue and cells

RNA was isolated from the collected tissues by placing approximately 50 mg of tissue in a LYSING matrix tube (MP Biomedical GmbH) containing 1 ml of RNA-BEE^{TM} on ice. Cells were disrupted in MagNA lyser instrument (Roche) (6,500 x *g*, 50 sec, and 20oC). Total RNA was extracted from the lysate using the RNA-Bee^{TM} isolation kit according to the instruction of the manufacturer. Contaminating DNA was removed by treatment of the RNA samples with DNase (1 U/µg of RNA, Fermentas). The quantity and purity of the isolated RNA was verified using a NanoDrop ND-1000 spectrophotometer. For isolation of RNA from cultured cells, 10^5 cells were collected in 250 µl of RNA-Bee^{TM} and directly subjected to the RNA-Bee extraction method described above. Purified RNA was stored in 25 µl of RNase-free water and stored at -80^{0}C until further analysis.

Quantitative real-time RT-PCR analysis

Quantitative real-time PCR on isolated RNA was routinely performed using the Reverse Transcriptase RT-qPCR Master Mix kit (Eurogentec). The reaction was performed in a Roche LightCycler®480 using 50 ng of DNase I-treated RNA samples as template and the primer sets and probes listed in Table 1. Probes for *IL-8 (CXCL8-CXCLi2)*, *IL-6*, *IL-1β* and *IFNβ* were labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5`-end and with the quencher N, N, N, N`- tetramethyl-6-carboxyrhodamine (TAMRA) at the 3`-end (Eurogentec). Inducible isoform of nitric oxide synthase (iNOS) transcripts were measured using the one-step RT-qPCR Master Mix Plus SYBR Green1 kit (Eurogentec). The following reaction conditions were used: Reverse Transcription step at 48^{0}C for 30 min followed by incubation for 10 min at 95^{0}C, 40 cycles of 15 s at 95^{0}C, and 60 s at 60^{0}C. Each sample was run in duplicate. Non-reverse transcriptase-treated samples, a template free sample, and a nuclease free water sample served as controls. Transcript levels were normalized to those for the chicken internal control genes glyceraldehyde-3-phosphate

dehydrogenase (*chGAPDH*) or glucose-6-phosphate dehydrogenase (*chG6PDH*, HD11 cells only). For each gene, results were expressed as fold change in mRNA level compared to the PBS-injected, GC-injected or *C. jejuni* challenged control group according to Schmittgen & Livak(52) using the formula: (1)Δ C_t target gene - Δ C_t GAPDH (or G6PDH) for each sample, (2) Δ C_t target gene treated - Δ C_t target gene control. The fold change for each gene transcript was determined using: Fold change = $2^{-\Delta(\Delta \text{ Ct gene treated } -\Delta \text{ Ct gene controll})}$. To calculate the relative expression levels for each of the bird groups, the transcript levels in samples of individual birds were compared with the mean value of the group of control birds, yielding a mean ± SEM value for the treated groups. The SEM values thus represent the variation in fold difference between individual chicken. The expression of the internal control genes was not influenced by the GC treatment.

Statistical analysis on all RT-qPCR results was performed on log transformed data the GraphPad Prism 6 multiple *t* test corrected for multiple comparison using the Holm-Šidák method, with alpha=5.000%

Nitric oxide assay

For measurement of nitric oxide (NO) production, the Griess assay was employed. In brief, cell supernatants collected after 17 h of incubation with the bacterial TLR agonist (or controls) were incubated (10 min, 20^{o}C, in dark) with an equal volume (50 µl) of Griess reagent 1 (1% sulfanilamide, Sigma-Aldrich). Then, an equal volume of Griess reagent 2 (0.1% N-naphthyl ethylene diamine dihydrochloride (Sigma-Aldrich) in 2.5% phosphoric acid) was added. After an additional 5 min of incubation in the dark, absorbance was measured at 550 nm in a spectrophotometer. The amount of produced NO was calculated from a calibration curve established by serial dilution (1-100 µM) of sodium nitrite ($NaNO_2$) in tissue culture medium. Data were analyzed using the GraphPad Prism 6 multiple *t* test. Values are expressed as the mean +/- SEM of at least three independent experiments.

Table 1. Primers and probes used in this study.

chGAPDH	Forward Reverse probe	GCCGTCCTCTCTGGCAAAG TGTAAACCATGTAGTTCAGATCGATGA (FAM)-AGTGGTGGCCATCAATGATCC-(TAMRA)
chIL-8 (CXCL8)	Forward Reverse probe	GCCCTCCTCCGGTTTCAG CGCAGCTCATTCCCCATCT (FAM)-TGCTCTGTCGCAAGGTAGGACGCTG(TAMRA)
chIL-1ß	Forward Reverse probe	GCTCTACATGTCGTGTGTGATGAG TGTCGATGTCCCGCATGA (FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)
chIFNβ	Forward Reverse probe	ACAACTTCCTACAGCACAACAACTA GCCTGGAGGCGGGACATG (FAM)-TCCCAGGTACAAGCACTG-(TAMRA)
chIL-6	Forward Reverse Probe	GCT CGC CGG CTT CGA GGT AGG TCT GAA AGG CGA ACA G (FAM)AGG AGA AAT ACC TGA CGA AGC TCT CCA- (TAMRA)
chiNOS	Forward Reverse	GGCAGCAGCGTCTCTATGACTTG GACTTTAGGCTGCCCAGGTTG
chG6PDH	Forward Reverse	CGGGAACCAAATGCACTTCGT CGCTGCCGTAGAGGTATGGGA

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Chapter 4

Glucose-sensitive regulation of the environmental pH by *Campylobacter jejuni*

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To be submitted

ABSTRACT

The local environmental conditions in the gut largely determine intestinal bacterial homeostasis. Here, we investigated the effect of bacterial metabolites and available nutrients on the environmental pH. Infection assays with different enteropathogens showed that the principle human foodborne pathogen *Campylobacter jejuni* did not influence the neutral environmental pH, whereas *Escherichia coli* and *Salmonella enterica* acidified the medium. Additional pH shift experiments revealed that *C. jejuni* can actively elevate the environmental pH from 4.8 to 7.4 with a 24 h period. The alkalinization occurred in the presence of the amino acids L-serine or L-aspartate but not with their fermentation products pyruvate and fumarate. Measurement of ammonia release by the bacteria with a modified microplate-based fluorometric technique indicated sufficient ammonia production by *C. jejuni* to neutralize the acidic environmental pH dependent on amino acid availability. Remarkably, the presence of glucose largely prevented the release of ammonia and the elevation of the pH, although *C. jejuni* cannot utilize glucose as energy source. Other sugars (galactose, fructose, and maltose) did not inhibit the production of ammonia and the elevation of the external pH. We conclude that *C. jejuni* is capable to actively maintain a neutral environmental pH via the glucose-sensitive release of ammonia.

INTRODUCTION

Metabolic interplay between bacterial species and molecular cross-talk between bacteria and the host are important for a healthy gut ecosystem (1). Many bacterial communities can adapt to the local environments encountered the host. These niches may differ in, for instance, available nutrients, atmospheric conditions, and the absence or presence of competitive or beneficial bacterial species (2). One important aspect in the bacterial adaptation process is the cytoplasmic and periplasmic pH homeostasis that permits bacterial survival and growth under acidic or alkaline external conditions (3, 4, 5, 6, 7, 8). Existing pH adaptation systems often involve pH sensing mechanisms and (post)transcriptional responses that alter cell structure, metabolism, or membrane transport systems related to the availability of oxygen, hydrogen, CO_2, urea, carbohydrate and amino acids (9, 10, 11, 12, 13). The adaptive changes can influence the relative magnitudes of the transmembrane electrical potential $\Delta\psi$ and the transmembrane ΔpH that together determine the proton motive force needed for energy production (4). Apart from ensuring pH homeostasis in individual bacterial cells, bacterial communities may increase their survival by active modulation of the environmental pH. A good example here is the gastropathogen *Helicobacter pylori* that can survive in the acidic environment of the stomach due to urease-dependent hydrolysis of urea to carbon dioxide and ammonia. Such type of adaptation mechanisms may reduce the need for continuous adaptation of individual bacteria to an unfavorable acidic or alkaline environment and may provide a competitive advantage in complex bacterial ecosystems.

The major bacterial human foodborne pathogen *Campylobacter jejuni* (*C. jejuni*) can rapidly colonize the gut of humans and warm-blooded animals. The bacterium occupies the chicken ceca with 1-2 days and reaches colonization levels of 10^9 bacteria per gram feaces without significant pathology. Colonization of humans often results in a serious enterocolitis with overt acute diarrhea, fever, nausea and headache. In a small number of cases, the infection may be followed by auto-immune paralysis (14, 15). One factor contributing to the successful colonization of the intestine by *C. jejuni* is the presence of flagella-driven motility that together with the spiral shape of the bacteria enable penetration of the mucus layer (16). Another factor is the abundant presence of the amino acids serine, aspartate, glutamate and proline in the chicken ceca. *C. jejuni* metabolism preferably utilizes these amino acids as carbon and energy source, although certain strains may also

utilize asparagine and glutamine (17). In contrast to many other enteropathogens *C. jejuni* cannot metabolize glucose with exception of a subset of *C. jejuni* subsp. *doylei* strains (18).

Despite the extensive knowledge about the genetic and metabolic adaptation of individual *C. jejuni* cells in response to changing environmental conditions, knowledge of the influence of *C. jejuni* of its microenvironment is scarce. Dependent on the environmental niche, *C. jejuni* may release metabolites like succinate and acetate and utilize molecules such as oxygen, hydrogen, formate or nitrate (19, 20, 21, 22). An important factor that may influence the colonization success of *C. jejuni* is the pH of the local microenvironment. In the present study we investigated the effect of *C. jejuni* on the external pH. Using a series of growth conditions and pH shift experiments, we discovered that *C. jejuni* but not *Escherichia coli* (*E. coli)* and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) stabilizes the environmental pH via a glucose-sensitive regulation of the production of ammonia (NH_3/NH_4^+). The produced ammonia can counteract the acidification of the milieu caused by glucose-utilizing bacteria.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma-Aldrich, unless stated otherwise.

Bacteria

Campylobacter jejuni strains 81116 (NCTC11828), NCTC 11168 and 81-176 (23, 24, 25, 26) were routinely cultured on blood agar base II medium plates (Oxoid Ltd., London, UK) containing 5% saponin lysed horse blood (Biotrading) at 37^{o}C under a microaerophilic atmosphere of 10% CO_2, 5% O_2, and 85% N_2. Planktonic cultures were grown under the same atmospheric conditions in 5 ml of Heart Infusion (HI) broth (Oxoid) in 25 cm^2 tissue culture flasks on a gyratory shaker (200 rpm). *Helicobacter hepaticus* strain ATCC 51448 was grown in the (HI broth in an atmosphere of 8.5% CO_2, 5% O_2, 7% H_2, and 79.5% N_2). For use in infection assays and pH shift experiments, bacteria (16 h of growth) were collected by centrifugation (5,500 x *g*, 10 min), rinsed with 3 x 1 ml of PBS (pH 7.2), resuspended in PBS, and added to tissue culture dishes filled with the appropriate tissue culture medium (DMEM, RPMI1640) (Life Technologies) or Hepes buffer saline (20 mM

Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) with the desired supplements.

E. coli NCTC 86 and *S.* Enteritidis strain 90-13-706 were grown in Luria-Bertani medium (Biotrading) at 37°C in air. After 16 h of growth, bacteria were collected by centrifugation (4,000 x *g*, 5 min), and prepared for use in the different assays as described above.

Chicken HD11 and NCSU macrophages (27, 28) and human HeLa-57A cells (29) were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% fetal calf serum (FCS) at 5% CO_2 at 37°C.

Infection assays

For use in infection assays, bacterial suspensions (10^5-10^9 CFU) were added to cultured eukaryotic cells kept in DMEM + 5% FCS and incubated at 37°C at 5% CO_2 in air. The pH of the medium was visually monitored using the pH indicator phenol red and quantified by using a two point calibrations Horiba B-712 (Laqua twin, Japan) pH meter or (for larger volume) a pHm210 apparatus (Meterlab). Bacterial growth during the assay was estimated from measurement of the optical density of the medium using a spectrophotometer (Thermo Electron Corporation) and by colony counting at 24-48 h after agar plating of serial dilutions of the culture supernatant.

pH shift experiments

The effect of bacteria on the environmental pH was tested using the identical setup as described for the infection assays but without the presence of eukaryotic cells. In addition, bacteria were added to media or buffer with a start pH of 4.3-7.0 rather than 7.4. Added supplements included L-serine, L-aspartate, glucose, maltose, galactose, fructose, sodium pyruvate, and sodium fumarate at the indicated concentrations. Bacteria were also grown in 5 cm dialysis tubes (22 mm diameter) with a 3 kDa cut-off (Snake skin Dialysis Tubing, Thermo Scientific). After filling of the tubes with the desired bacterial suspension, the clipped tubes were bathed in 5 ml of medium in a 9 mm Petri dish, and incubated for the indicated times at 37°C at 5% CO_2 in air. The pH of the media was determined as described above.

Ammonia measurement

The detection of ammonia in the media and buffers was based on orthophthaldialdehyde-fluorescence determination method (30, 31). Briefly, a solution of working reagents (WR) was prepared that was composed of 21 mM of borate buffer (pH 9.5), 0.063 mM of sodium sulfite, and 0.4% of orthophthaldialdehyde (OPA) dissolved in ethanol. The solution was protected from light. For measurement of ammonia levels, buffer or media samples were cleared by centrifugation (8,000 x g, 5 min) and transferred (50 µl) to a 96-well plate (Corning, USA). Then, 100 µl of Hepes solution (20 mM Hepes, with/ without glucose, pH 7.4) was added and then gently mixed with 50 µl of WR (in the dark). Each 96-well plate was also loaded with serially dilutions (1.5-400 µM) of a standard solution of ammonium hydroxide in deionized water to quantify the measured ammonia levels. Samples to which the equivalent volume of borate buffer instead of WR solution was added served as background control. To exclude fluorescence reactions of OPA with substances in the samples that may alter the intensity of the fluorescence (matrix effect, MF), samples with an equivalent volume of WR were added to the same volume of deionized water or hepes buffered saline. When all wells were filled, the plate was wrapped in aluminum foil and incubated at room temperature for 4 hours in the dark. The fluorescence intensity was measured in a FluoStarOmega apparatus (Promega) at an excitation wavelength of 380 nm and emission wavelength 505 nm. Fluorescence matrix effect values were subtracted from the fluorescent sample values as the background values were generally below. Standard ammonia dilution series, 4 wells to determine matrix effects, and 2 wells to indicate background values were present on each 96-well plate.

Statistical analysis

GraphPad Prism 6.05 software was used for all statistical analyses. Data were analyzed by multiple *t*-test for comparison of two groups using the Holm-Šidák method. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Campylobacter jejuni *stabilizes the environmental pH*

Infection assays in which different bacterial enteropathogens were incubated with cultured chicken macrophages showed that *E. coli* NCTC 86 and *Salmonella enterica* serovar Enteritidis strain 90-13-706 caused a rapid acidification of the culture medium (Fig. 1A). Extensive growth of *C. jejuni* strain 81116 in same medium (DMEM + 5% FCS) did not lower the pH over a period of up to 24 h (Fig. 1A). The different effects of the bacteria on the medium pH occurred with different tissue culture media (DMEM, RPMI1640) and host cell types (epithelial cells, fibroblasts, macrophages). Similar experiments in the absence of eukaryotic cells yielded similar results (data not shown). This indicated that the changes in medium pH most likely reflected differences in bacterial metabolism.

Although the effects of the bacteria on the environmental pH may easily be attributed to the ability of *E. coli* and *S.* Enteritidis but not *C. jejuni* to convert sugars into acetate, we performed a co-incubation of *E. coli, S.* Enteritidis and *C. jejuni*. To avoid overgrowth of *C. jejuni* by *E. coli* or *S.* Enteritidis, the strains were placed in dialysis tubes (3 kDa pore size) and incubated together in a Petri dish filled with sterile tissue culture medium containing the pH indicator phenol red. After 4 h of incubation (37^{o}C, 5% CO_2), the tubes containing *E. coli* and *S.* Enteritidis had turned yellow (pH < 6.8), while the tube containing *C. jejuni* retained its original red color (pH 7.4) (Fig. 1B). After 24 h incubation, the acidification of the medium had turned the entire dish yellow except for the tube containing *C. jejuni* (Fig. 1C), suggesting that *C. jejuni* can influence its environmental pH.

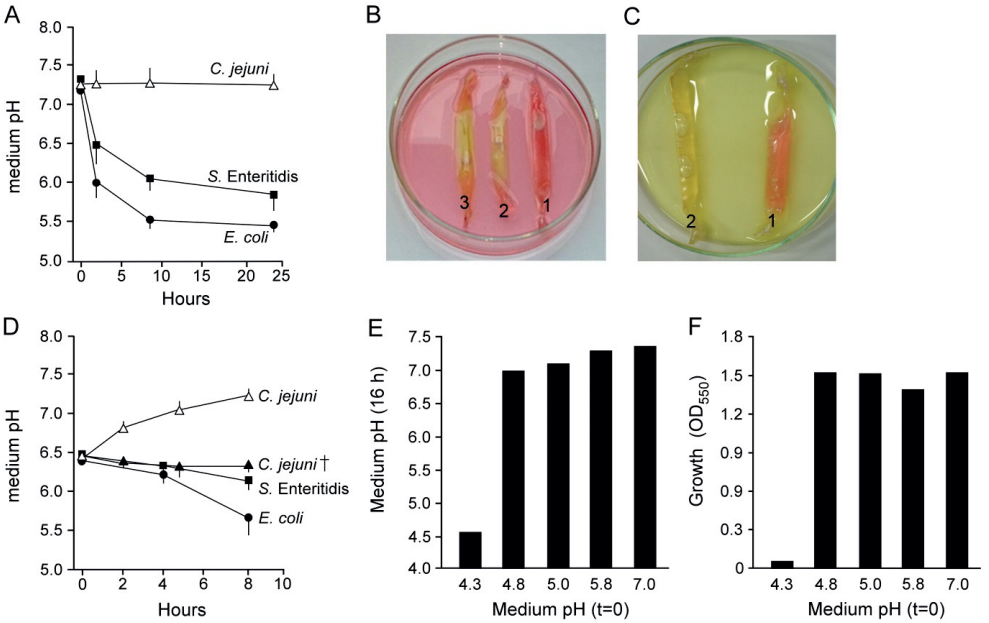

Figure 1. *Campylobacter jejuni* actively induces a neutral environmental pH. *C. jejuni* (A-F), *E. coli* (A-D) and/or *S.* Enteritidis (A-D) were incubated (0-24 h): (A) in the presence of chicken macrophages in DMEM plus 5% FCS; (B-D) in the absence of eukaryotic cells in DMEM pH 7.4 or 6.4; (E-F) in HI medium with a starting pH of 4.3 to 7.4. The pH of the medium (A, D, E) was measured using a pH meter. Bacterial density (F) was estimated using a spectrophotometer. Data (A, D) are the mean ± SEM of four experiments. Results depicted in E and F is representative of 3 experiments. Illustrations B and C show the visual change in medium pH after 4 and 24 h of growth of *C. jejuni* (1), *E. coli* (2) and/or *S.* Enteritidis (3) in dialysis tubes.

C. jejuni *actively neutralizes a low environmental pH*

To test the hypothesis that *C. jejuni* may actively regulate the medium pH, we adjusted the pH of tissue culture medium to 6.4 prior to the addition of bacteria. In the presence of *C. jejuni*, the pH of the medium started to rise within 2 h of incubation (37°C, 5% CO_2) and reached a pH value of 7.2 after approximately 8 h of incubation (Fig. 1D). In the absence of *C. jejuni* or after the addition of heat-killed *C. jejuni*, the low pH of the medium did not change, indicating that viable *C. jejuni* are required for the effect. The addition of the *E. coli* and *S.* Enteritidis did not elevate the pH but caused even further acidification of the medium (Fig. 1D). The ability of *C. jejuni* to neutralize the acid pH was detected for medium with an initial pH value of 4.8 and higher (Fig. 1E). In this environment *C. jejuni* showed efficient growth (Fig. 1F). At a medium pH of 4.3, *C. jejuni* was unable to raise the

pH and also showed no growth. These data strongly suggest that C. *jejuni* is capable to actively elevate the environmental pH.

Amino acids are required for* C. jejuni *to neutralize the environmental pH

To identify the pH neutralizing factor(s) released by *C. jejuni*, we used Hepes buffered saline of pH 7.4 or 6.4, enriched with different supplements. Without nutrients, *C. jejuni* lowered the medium pH of 7.4 and 6.4 (Fig. 2A and D). A similar drop in pH was observed for *E. coli* and *Salmonella* when added to a medium of pH 7.4 (Fig. 2B and C). When added to medium of pH 6.4, no further drop in pH value was observed (Fig. 2E and F). When glucose (2%) was present as a supplement, the pH of the buffer containing *C. jejuni* showed no additional changes, consistent with its inability to metabolize this sugar (data not shown). Addition of glucose to the *E. coli* and *S.* Enteritidis cultures caused a further drop in pH (Fig. 2B-F), probably due to the release of acetate into the medium.

When the amino acid L-serine (2 mM) was added as sole carbon and energy source (Fig. 2D), the pH of the *C. jejuni* culture was stable between values 7.2 and 7.4 for up to a period of 24 h (Fig. 2A). Moreover, *C. jejuni* was capable to elevate the pH from 6.4 to 7.2 when L-serine was present (Fig. 2D). This effect of L-serine was not observed for *E. coli* and *S.* Enteritidis (Fig. 2B-F). As *C. jejuni* hydrolyzes L-serine to pyruvate plus ammonia, we also tested the effect of pyruvate. The addition of pyruvate (2 mM), which supported *C. jejuni* growth (data not shown), was not sufficient to elevate the pH of the medium (Fig. 2D). The pH neutralizing effect of L-serine was also observed after the addition of 2 mM of L-aspartate (see below) which can be converted to fumarate plus ammonia. The addition of fumarate was not sufficient to stabilize the external pH (Fig. 2A), although *C. jejuni* utilized fumarate for bacterial growth (data not shown). Overall, these findings indicate that *C. jejuni* requires amino acids to neutralize the environmental pH.

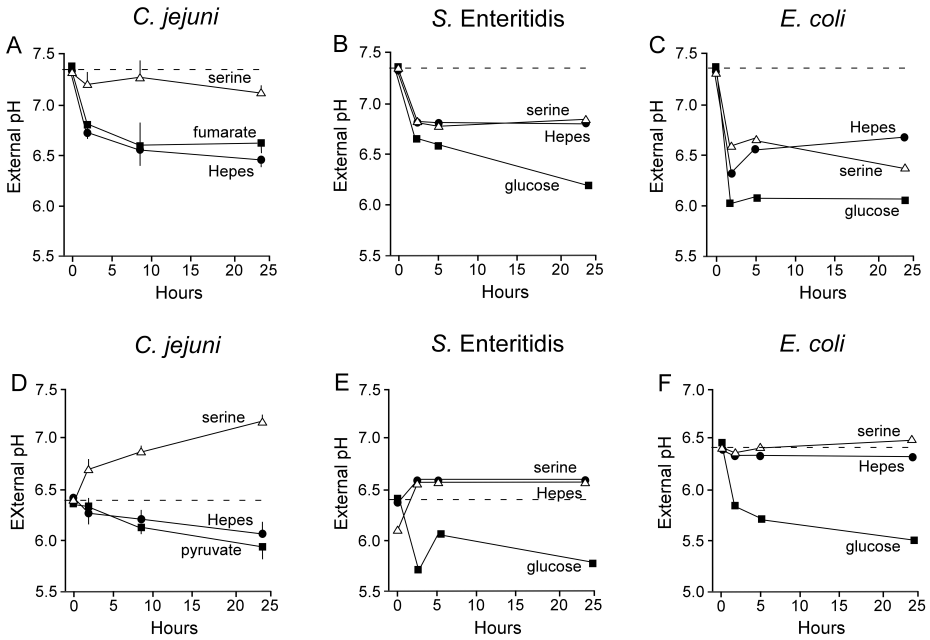

Figure 2. Effect of L-serine and glucose on external pH. The indicated bacterial species were incubated (0-24 h) in Hepes buffered saline supplemented with 2 mM L-serine, 5 mM glucose, 2 mM fumarate, or 2 mM pyruvate and with starting pH values of 7.4 (A-C) or 6.4 (D-F) indicated with the dotted line. The pH of the buffer was measured at the indicated times. Results are the mean ± SEM of five experiments.

C. jejuni *release ammonia into the medium*

As deamination of both L-serine and L-aspartate by *C. jejuni* is accompanied with the formation of ammonia (NH_3), we tested whether *C. jejuni* was capable to release ammonia into the medium under the conditions employed. *C. jejuni* was added to Hepes buffer supplemented with L-serine, L-aspartate or the derivates pyruvate and fumarate. The ammonia concentration in the buffer was measured at different time points using the orthophthadialdehyde (OPA) method. *C. jejuni* caused a rapid release of substantial amounts of ammonia into the medium in the presence of L-serine or L-aspartate but not pyruvate or fumarate (Fig. 3A). Similar experiments with *E. coli* and *Salmonella* showed that, in comparison of *C. jejuni*, both species released, even the absence of glucose, only minor amounts of ammonia in the presence of L-serine or fumarate during the 24 h incubation period (Fig. 3B and C).

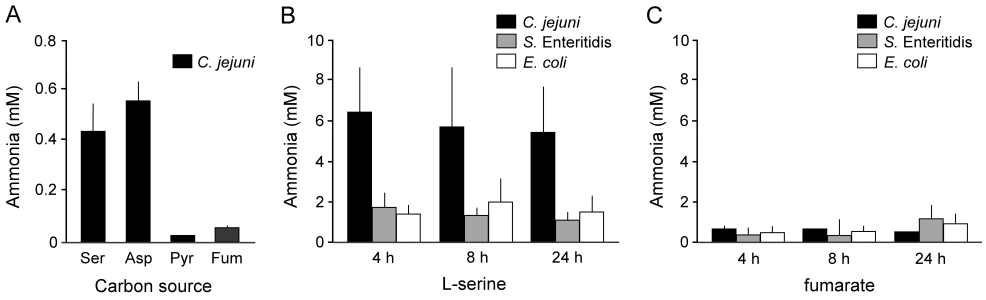

Figure 3. Ammonia release by *C. jejuni.* (A) *C. jejuni* strain 81116 ($2x10^6$ CFU ml^{-1}) was incubated (microaerophilic conditions, 4 h, 37ºC) in Hepes buffer supplemented with 2 mM of L-serine. L-aspartate, pyruvate or fumarate. Ammonia levels in the supernatant were measured using the OPA method. Data are the mean ± SEM of three experiments. (B-C) *C. jejuni, S.* Enteritidis and *E. coli* ($2x10^6$ CFU ml^{-1}) were incubated in Hepes buffer supplemented with 5 mM of (C) L-serine or (D) fumarate and net ammonia release was measured during a 24-h period. Data are the mean ± SEM of at least five experiments.

C. jejuni *ammonia release is sufficient to neutralize the environmental pH*

In order to ascertain that the amounts of ammonia released by *C. jejuni* are capable to account for the observed neutralization of the acidic environmental pH, we first titrated acidified hepes buffer (pH 5.6) with ammonia. Results showed that approximately 12 mM of ammonia was needed to increase the pH of the buffer to a value of pH 7.4 (Fig. 4A). When tissue culture media (DMEM, RPMI1640) were used instead of the Hepes buffer, much less ammonia (1-2 mM) was needed to neutralize the low pH (Fig. 4A) probably due to the lower buffering capacity of the tissue culture media compared to Hepes buffer.

To estimate the amounts of ammonia released by *C. jejuni* in the presence of L-serine, we incubated *C. jejuni* with a range of L-serine concentrations and measured the released ammonia after 4 hours. Ammonia concentrations directly correlated with the amounts of L-serine in the buffer. At 1 mM of L-serine approximately 1 mM of ammonia was produced within 4 h, while at 8 mM of L-serine more than 7 mM of ammonia was detected (Fig. 4B). As these levels are more than sufficient to neutralize the environmental pH of tissue culture media, ammonia is likely a major factor contributing to the regulation of the environmental pH by *C. jejuni*.

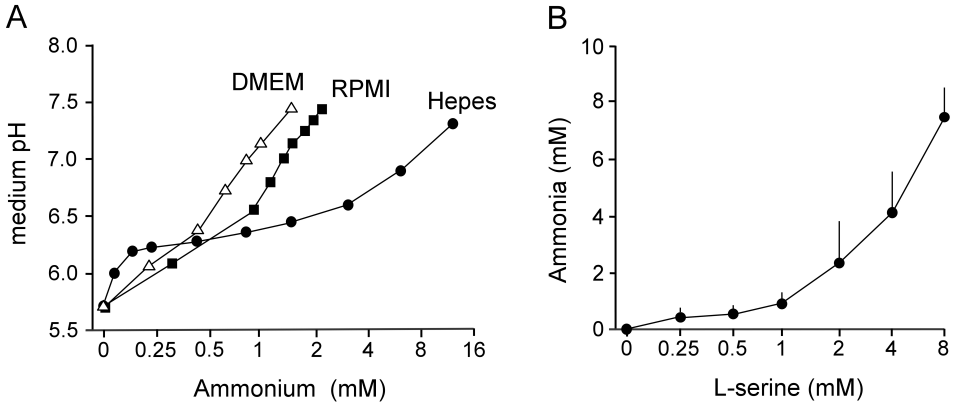

Figure 4. Adjustment of the medium pH by ammonia. (A) Ammonia was added to low pH tissue culture medium and hepes buffered saline (pH 5.6) and the change in pH was determined using a pH meter. Data are representative of three experiments. (B) *C. jejuni* strain 81116 ($2x10^6$ CFU ml^{-1}) was added to Hepes buffer supplemented with the indicated concentrations of L-serine. The amount of ammonia in the medium was determined after 4 h of incubation using the OPA-method. Data are the mean ± SEM of three experiments.

***Glucose inhibits ammonium release by* C. jejuni**

In search for key nutrients that enable *C. jejuni* to change its environmental pH, we noticed to our surprise that adding the combination of L-serine (2 mM) and glucose (2%) to *C. jejuni* kept in Hepes buffer (pH 6.4) prevented the elevation of pH that was observed after the addition of L-serine alone. This effect was noted for the both *C. jejuni* strains tested (81116 and 81-176) (Fig. 5A and B). Comparison of different types of carbohydrates, i.e. glucose, galactose, fructose and maltose, alone and in combination with L-serine indicated glucose as the most effective inhibitor of the neutralization of the low pH by *C. jejuni* (Fig. 5A and B). None of the different sugars influenced the *C. jejuni* growth (data not shown). The effect of glucose was observed both when incubation was carried out at the body temperatures of humans (37^oC) or chicken (42^oC).

Measurement of ammonia levels in the media showed that glucose but not the other tested sugars prevented the release of high concentrations of ammonia in the presence of L-serine (Fig. 5C). The inhibition was dependent on the amount of glucose added and was also observed when L-serine was replaced by L-aspartate as the source of the ammonia (Fig. 5D and E). Repetition of the experiment with the two *C. jejuni* strains and the distantly related *Helicobacter hepaticus* confirmed the inverse relationship between the amount of added glucose (0.5-5%) and the concentration of ammonia in the medium

produced from L-serine (Fig. 5F). Based on the titration results (Fig. 4), the low concentrations of ammonia that were detected in the buffer in the presence of glucose (<100 µM) seemed insufficient to elevate the low pH. The inhibitory effect of glucose on the elevation of the medium pH may imply that this process is mainly active under conditions of high glucose utilization (e.g. by other enteropathogens).

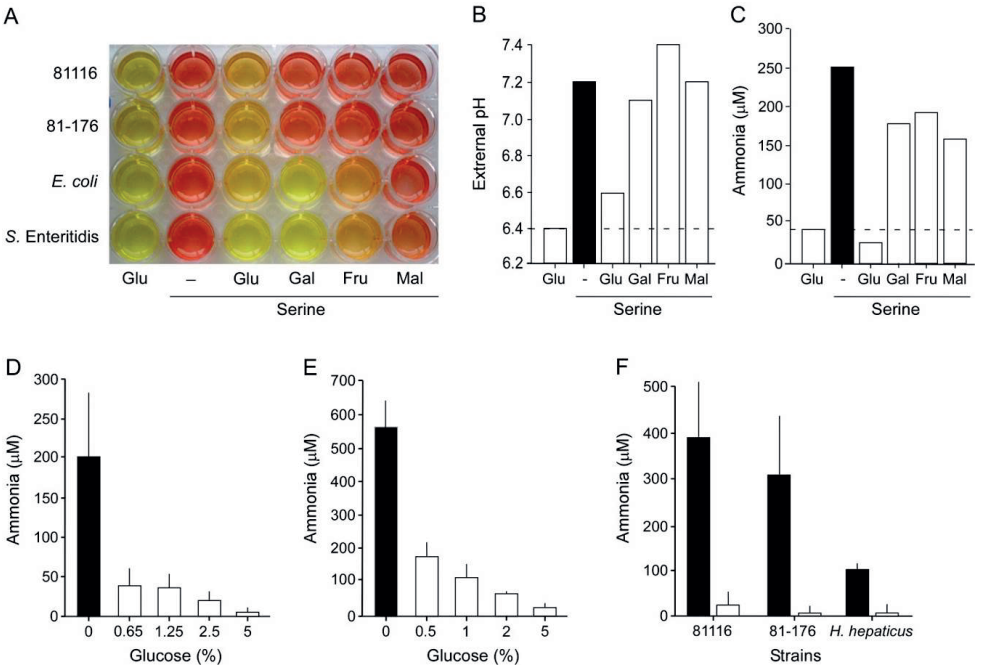

Figure 5. Glucose inhibition of *C. jejuni* ammonia release and adjustment of the external pH. (A-C) *C. jejuni* strains 81116 and 81-176 were incubated (24 h, microaerophilic conditions) in HI (pH 6.4) supplemented in the absence and presence of 2 mM L-serine and/or 5% of glucose (Glu), galactose (Gal), fructose (Fru) and maltose (Mal). The medium pH was (A) visually followed using the pH indicator phenol red (shown at 24 h) and (B) quantified with a pH meter. (C) Ammonia concentrations (4 h) were determined using the OPA method. Similar incubations with *E. coli* and *S.* Enteritidis served as controls. Results shown are representative of three experiments.

(D-E) Ammonia release by *C. jejuni* strain 81116 after 4 h of incubation in Hepes buffered saline (pH 7.4) supplemented with 2 mM of (D) L-serine or (E) L-aspartate and the indicated concentrations of glucose. (F) Glucose inhibition of ammonia production (4 h, 37°C) in *C. jejuni* strains 81116 and 81-176 and *H. hepaticus*. Experiments were repeated at least three times and mean values ± SEM are shown.

DISCUSSION

In the present study we demonstrate that *C. jejuni* but not *E. coli* or *S.* Enteritidis releases large amounts of ammonia into the environment and is capable to actively maintain a neutral environmental pH. Both effects require bacterial access to the preferred amino acids L-serine or L-aspartate and do not occur with their deamination products pyruvate or fumarate. The neutralization of the low pH can be mimicked by the addition of ammonia. Remarkably, the release of ammonia by *C. jejuni* and the elevation to a neutral external pH are blocked in the presence of glucose, despite the fact that *C. jejuni* cannot metabolize this sugar. Together, the present results for the first time indicate that *C. jejuni* has evolved a glucose-sensitive pH adaptation system to influence the environmental pH in addition to the well-established rapid regulation of cytoplasmic pH homeostasis. The identified mechanism may serve to counteract the acidification of the microhabitat caused by glucose-utilizing bacteria.

The initial observation that *C. jejuni* does not acidify tissue culture medium in contrast to *E. coli* and *Salmonella* Enteritidis is typically attributed to the difference in glucose utilization between these species. Numerous studies indicate that *C. jejuni* is not capable to metabolize glucose, whereas the fermentation of glucose by *E. coli* and *S. enterica* results in the release of mainly acetate that rapidly acidifies the medium. Our first evidence that *C. jejuni* may actively regulate the environmental pH was its ability to elevate a low pH from as low as 4.8 to 7.4. This effect required viable bacteria and the presence of amino acids (like L-serine or L-aspartate) that are used as major carbon and energy source by the pathogen. The importance of amino acid utilization in this process is underpinned by the absence of the pH neutralizing effect when non-viable bacteria were added or when pyruvate or fumarate were present a single energy source. With these carbon sources, *C. jejuni* grew very well but did not elevate the low pH. As the formation of pyruvate and fumarate from L-serine and L-aspartate is accompanied by the production of ammonia, we hypothesized that the bacterial release of ammonia may influence the environmental pH. Measurement of ammonia concentrations in the medium confirmed that in the presence of L-serine and L-aspartate *C. jejuni* released sufficient amounts of ammonia to adjust the environmental pH.

The production of ammonia is a common trait of many prokaryotes. In most bacterial species, ammonia is produced during the deamination of amino acids or as product of the respiratory nitrate-nitrite-ammonification pathway (32, 33, 34). The gastric pathogen *Helicobacter pylori* and the related *Helicobacter hepaticus* release large amounts of ammonia into the environment *via* their periplasmic urease enzyme that hydrolyzes urea to ammonia and carbon dioxide (35, 36). This activity is crucial for bacterial pathogenesis (37, 38). *C. jejuni* lacks the urease enzyme but is believed to form cytosolic ammonia via the hydrolysis of amino acids as well as periplasmic ammonia via the respiratory NapA-NrfA-ammonia route (39, 40). In *E. coli* the concentration of intracellular ammonia depends on the diffusion of the gaseous NH_3 across the bacterial membrane and the uptake of NH_4^+-NH_3 for use in amino acid (mainly glutamate) synthesis (41). The relative contribution of the amino acid hydrolysis and ammonification pathways of *C. jejuni* to the measured environmental ammonia concentrations is unknown. Nevertheless, our finding that ammonia is produced with L-serine or L-aspartate as only available nutrients suggests that the deamination activity is sufficient to relieve the low external pH.

Perhaps the most unexpected finding in our study is inhibition of the ammonification of the medium and the concomitant rise in external pH in the presence of glucose. It is generally accepted that *C. jejuni* cannot metabolize glucose due to the absence of 6-phosphofructokinase and enzymes from the oxidative branch of the pentose phosphate and Entner-Doudoroff pathways. Certain *C. jejuni* subsp. *doylei* isolates contain a genomic island encoding enzymes involved in glucose catabolism (18) but these genes have thus far not been found in non-doylei *C. jejuni* strains. To our knowledge, uptake of glucose by *C. jejuni* has never been demonstrated, although a subset of strain can catabolize L-fucose (42, 43, 44). Galactose, fructose or maltose could not replace glucose as inhibitors of the release of ammonia. This finding suggest a specific, perhaps promiscue interference of glucose with an as yet undefined metabolic regulatory process that directly or indirectly influences the ammonia release by *C. jejuni*.

The finding that glucose influences the ammonia release and the effect on the environmental pH of *C. jejuni* may be of particular interest in the natural ecosystem of the gut. It can be imagined that *in vivo C. jejuni* shapes its own niche with a neutral environmental pH and this way counteracts the strong acidification of the microenvironment caused by saccharolytic bacteria like *E. coli* and *S.* Enteritidis. The

glucose-sensitivity of the external pH regulation may ensure that the system is most active when glucose utilization and hence environmental acidification by other bacteria is high. The preservation of a neutral external pH may alleviate the need and expenditure by each *C. jejuni* bacterium of continuous activity to react to acid stress and to control internal pH homeostasis. Under acidic conditions, *C. jejuni* shows strongly altered expression and protein profiles (45, 46, 47), indicating major and probably costly efforts of bacterial adaptation. A separate, additional advantage of the release of ammonia by *C. jejuni* may be its effect on bacterial pathogenesis. Ammonia produced by *H. pylori* has been suggested to promote colonization of the mucus layer (48). Ammonia also influences the status of the tight junctions between cells that regulate the integrity of the intestinal barrier (49). Once inside cells, ammonia is known to be able to inhibit the acidification of the late endosomal pH (50). This way *C. jejuni* may theoretically block its delivery to endo-lysosomes and thus enhance its intracellular survival. However, *C. jejuni* may utilize alternative strategies to escape this trafficking route (51).

In conclusion, the present study for the first time shows that *C. jejuni* is capable to actively influence the micro-environmental pH via the glucose-sensitive release of ammonia. This bacterial strategy may reduce the need for continuous adaptation of individual bacteria to survive a suboptimal external pH. Whether the released ammonia influences *C. jejuni* pathogenesis awaits further investigation.

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Chapter 5

Campylobacter jejuni modulates the innate immune response by altering protein S-nitrosylation

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ABSTRACT

Nitric oxide is a major cellular messenger (NO) that regulates a multitude of cellular events through the reversible S-nitrosylation of signaling molecules and transcription factors. In addition, nitric oxide is a potent bactericidal compound. Several bacterial species including *Campylobacter jejuni* detoxify or even utilize NO. We hypothesized that the utilization of NO by *C. jejuni* alters eukaryotic protein S-nitrosylation and thereby modulates the host cell immune response. To test the hypothesis, we incubated chicken macrophages with LPS, *C. jejuni*, and/or the exogenous NO donor GNSO. The protein S-nitrosylation status of the eukaryotic cells was determined using the biotin-switch method. Intracellular NO was visualized by microscopy using a trappable fluorescent NO probe. Effects on chicken cytokine gene expression were determined by quantitative RT-PCR. Results showed that the addition of exogenous NO enhanced S-nitrosylation of proteins in chicken immune cells and inhibited LPS-induced cytokine expression. Utilization of NO by wild type C. *jejuni* reversed the protein S-nitrosylation and restored the LPS-induced gene expression. This effect was not observed with a *C. jejuni*ΔNrfA mutant that was not capable to scavenge NO. Our results indicate that *C. jejuni* can modify the innate immune response by scavenging host-derived NO resulting in a change in S-nitrosylation.

INTRODUCTION

Nitric oxide (NO) is a widespread messenger molecule that participates in key physiological processes such as endothelial cell function, blood flow, cell signaling, and immune activation (1, 2, 3). NO is produced by different vertebrate cell types but can also be derived from exogenous sources. The main endogenous source of NO during inflammation is activated macrophages (4, 5, 6). These cells express an inducible nitric oxide synthase (iNOS) when activated. During infection this enzyme generates large amounts of NO during the O_2-dependent conversion of L-arginine to L-citrulline (7, 8, 9, 10). The main exogenous source of NO is dietary nutrients that enter the dietary nitrate-nitrite-NO entero-salivary route (11). This pathway involves the transport of nitrate through the intestinal mucosa into the bloodstream and the active secretion from the circulating nitrate into the saliva. After conversion into nitrite (up to 2 mM) by the oral microbiota, the swallowed nitrite converts to NO in the acidic stomach environment (12).

At the cellular level, NO exerts its effects through reversible reaction with several intracellular targets such as critical thiols in cysteine residues (13) and metal centers in proteins (haem, Fe-S clusters) (14, 15). Targets of S-nitrosylation include essential mediators of innate immune pathways (e.g. p60, p65, caspases) and other transcription factors (16, 17, 18, 19). The reversible modification of proteins influences their signaling activity. This explains the global effects of NO on a variety of cell processes including gene transcription, the innate host defense, and cancer development (20, 21, 22, 23, 24, 25, 26).

Nitric oxide and its reactive nitrogen species also play an important role in the defense against invading bacteria. NO is an uncharged gaseous molecule that readily diffuses across bacterial membranes before it is oxidized to nitrite. NO is toxic to bacteria through inhibition of key metabolic enzymes such as terminal cytochromes in the electron transport chain. This results in a lack of energy and an arrest of bacterial DNA and protein synthesis. The role of NO and nitrous compounds in the defense against bacterial pathogens is illustrated by the increased/high susceptibility of iNOS knockout (*nos2*$^{-/-}$) mice to bacterial infections (27).

Distinct bacterial species have evolved different strategies to resist high concentrations of NO and even exploit NO to their own benefit. The common principle is that NO is detoxified either by oxidation or, when oxygen is limited, by reduction to nitrous

oxide (N_2O) or ammonia (28, 29). The initial detoxification step often involves the binding of NO to bacterial haemoglobins (30). These NO-binding proteins, which may be induced in response to nitrosative stress, catalyze the conversion of NO and O_2 to nitrite and act as a terminal electron acceptor during microaerobic respiration (31). This way host-derived NO can even provide a growth advantage to nitrate proficient bacteria such as *E. coli* and *Campylobacter jejuni* (32, 33, 34, 35). In *Neisseria meningitidis* and several other Gram-negative bacterial species, NO is detoxified through bacterial denitrification. In this process, the nitrate oxide reductase complex (Nor) reduces NO to nitrous oxide and dinitrogen gas that can supplement bacterial growth in a low oxygen environment (36, 37, 38).

The important role of NO in host cell signaling but also the consumption of NO by certain pathogens led us to investigate whether bacteria may exploit metabolic cross-talk as a strategy to modulate host cell biology. We tested this hypothesis using the principal human foodborne pathogen *Campylobacter jejuni* which has the chicken gut as main reservoir. *C. jejuni* is regarded as an obligate microaerophilic bacterium that can utilize nitrate and nitrite as terminal electron acceptor in a low oxygen environment via the periplasmic nitrate and nitrite oxidases NapA and NrfA (39, 40). *C. jejuni* can counteract inhibition of its aerobic respiration by NO via the single-domain globin Cgb but also detoxify NO via the constitutively expressed NrfA protein complex (41). This complex typically catalyzes the conversion of nitrite to ammonia but can also use NO as a substrate (42, 43).

In the present study we determined the effect of exogenous NO as well as host-derived NO on *C. jejuni* viability, the consumption of NO by *C. jejuni*, its impact on the S-nitrosylation of host proteins and on the host innate immune system. Evidence is provided that the scavenging of NO by *C. jejuni* changes the *S*-nitrosylation status of host proteins and perturbs the local TLR-activated inflammatory response.

MATERIALS AND METHODS

Cell culture

The HD-11 and MQ-NCSU chicken cell lines (44, 45) were routinely grown (5% CO_2, 37^oC) in 25 cm^2 tissue culture flasks (Nunc) in 5 ml of Dulbecco's modified Eagle's medium DMEM (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum

(FBS). For use in experiments, cells were seeded in 6- or 12-wells tissue culture plates at 24 h prior to use.

Reagents

S-nitrosoglutathione (GSNO), sodium-L-ascorbate, N-methyl-L-arginine acetate salt, IGEPAL CA-630, aminoguanidine hydrochloride, S-methylmanethiosulfonate, streptavidin-agarose, monoclonal anti-biotin antibody clone BN-34, mercury (II) iodide and neocuprine were purchased from Sigma Aldrich. EZ-link HPDP-biotin and Pierce avidin agarose were purchased from Thermo scientific. Protease inhibitor cocktail was obtained from Roche and nor –NOHA from Bio-connect. The nitric oxide sensor (intracellular) kit (FL2E) was purchased from STREM chemical, USA.

Bacteria

Campylobacter jejuni strain 81116 (NCTC11828) and its derivatives 81116ΔNrfA and 81116ΔNapA were routinely grown on blood agar base II medium (Oxoid Ltd., London, UK) with 5% saponin lysed horse blood (Biotrading) at 37^{o}C under a microaerophilic atmosphere of 10% CO_2, 5% O_2, and 85% N_2 or in Heart Infusion (HI) broth (Oxoid). When appropriate, growth media were supplemented with kanamycin (50 μg ml^{-1}). Planktonic cultures were grown (37^{o}C, 17 h) in 5 ml of HI broth in 25 cm^2 flasks placed in a jar with a microaerophilic atmosphere (10% CO_2, 5% O_2, and 85% N_2) on a gyratory shalker (200 rpm). Starter cultures were used to inoculate 5 ml of HI broth (OD_{550}: 0.005) to obtain exponentially growing bacteria (37^{o}C). For use in experiments, bacteria (OD_{550}: 0.5) were collected by centrifugation, washed twice and resuspended in Hepes buffered saline (20 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4).

Construction of* C. jejuni *mutants

The *nrfA* (CJ_1357c) and *napA* (CJ_1358c) genes with their flanking sequences were amplified by PCR using the primer sets listed in Table 1 using genomic DNA of strain 81116 as a template. The PCR products were cloned into the pJET1.2 vector (Thermo) creating pJET*nrfA* and pJET*napA*. Plasmid pJET*nrfA* was digested with *Bam*HI to delete 240 bp and introduce the chloramphenicol resistance cassette (Cm^R) from pAV35. Inverse PCR using pJET*napA* as template was used to delete 1.5 kb from *napA* and to create the

unique restriction site *Bam*HI used to introduce the kanamycin resistance cassette (Km^R) from pMW2. The resulting knockout plasmids carrying the resistance cassettes in the same orientation as the inactivated gene was used to transform *C. jejuni* 81116 by natural transformation, yielding the mutants 81116ΔNrfA and 81116ΔNapA. Disruption of the genes was confirmed by PCR.

Table 1. Primers used for the construction of *C. jejuni* 81116ΔNapA and 81116ΔNrfA.

NapA gene fw	TTCCAAAGCTGCTGTAGATGAG
NapA gene rev	ATGCAGCCTTAAAGGCACTAAC
NrfA outward BamHI fw	CGC**GGATCC**CACCAAGCATAGAGCTTGGAACAC
NrfA outward BamHI rev	CGC**GGATCC**CATCAACCCAAAACCAGGCTCTCC

Restriction sites are indicated in bold letters.

Nitric oxide assay

Nitric oxide released in the culture supernatant was estimated using the Griess assay, which measures nitrite resulting from the oxidation of NO. After centrifugation (5,500 x *g,* 10 min), 50 µl of culture supernatant was added to an equal volume of Griess reagent 1 (1% sulfanilamide), incubated (20^{o}C) for 10 min in dark, and then added to an equal volume of Griess reagent 2 (0.1% N-naphthyl ethylene diamine dihydrochloride in 2.5% phosphoric acid). After 5 min of incubation (20^{o}C), absorbance was measured at 550 nm using a Fluostar Omega (BMG Labtech) spectrophotometer. Absorbance values were converted to absolute values using the slope of a calibration curve established using serial dilutions (1.5-100 µM) of sodium nitrite ($NaNO_2$).

Protein S-nitrosylation assay

S-Nitrosylated proteins were detected using the biotin-switch assay method (46). Cultured cells were rinsed twice with DPBS and lysed in 0.4 ml of lysis buffer (25 mM Hepes, 0.1 mM EDTA, 50 mM NaCl, 1% IGEPAL CA-630, 0.5 mM PMSF plus protease inhibitor, pH 7.5). After centrifugation (4,000 x g, 15 min, 4^{o}C), the supernatant was collected and stored on ice. The protein concentration of the supernatant was measured using the BCA kit (Thermo scientific). Samples were diluted with lysis buffer to final protein concentration of

0.7 mg ml^{-1}. To block free thiols, 1.8 ml of HEN blocking buffer (100 mM Hepes, 1 mM EDTA, 0.1 mM Neocuprine, pH 8.0 plus 0.2 ml of 25% SDS along with 20 µl of 10% S-methyl methane thiosulfate) was added to 200 µl samples and incubated (50°C, 15 min, in the dark) with frequent shaking. Then, three volumes of iced acetone (-20°C) were added to precipitate proteins (-20°C, 20 min). The precipitate was collected by centrifugation (2,000 x g, 5 min, 4°C), rinsed three times with 70% pre-cold acetone, and dissolved in 0.24 ml of HENS buffer (HEN buffer supplemented with 1% SDS). Next, 30 µl of 200 mM (+)-sodium L-ascorbate (final concentration 20 mM) and 30 µl of biotin-HPDP (2.5 mg ml^{-1}) were added. An equivalent concentration of NaCl was used as an ascorbate-free control. After labeling for 90 min (20°C, in the dark), the proteins were precipitated with two volumes of pre-cold acetone. The pellet was briefly rinsed three times with 50% acetone and then dissolved in 0.25 ml of HENS/10 buffer (HEN buffer diluted 10-fold with distilled water) plus 0.75 ml of neutralization buffer (25 mM Hepes, 1 mM EDTA,100 mM NaCl, 0.5% Triton X-100, pH 7.5). The sample was then mixed with 30 µl of prewashed Pierce Avidin Agarose beads and gently rotated overnight at 4°C. All of the above manipulations were performed in the dark. The beads were collected by centrifugation (200 x g, 20s) and rinsed four times with rinsing buffer (25 mM Hepes, 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 7.7). Labeled SNO proteins were eluted from the beads by heating (95°C) in 50 µl of HEN buffer containing concentrated (6x) non-reducing Laemmli loading buffer, separated by SDS-PAGE, and either analyzed by silver staining or, after transfer to a nitrocellulose membrane, probed with monoclonal mouse Anti-Biotin antibody (1:1,000) and the appropriate conjugate. All experiments were repeated at least three times with comparable results.

Cell activation assays

To analyze the innate immune response under different experimental conditions, cells were grown overnight to nearly confluence in 12-well plates in 5 ml of DMEM + 5% FCS. Prior to start of the assay, the medium was replaced with fresh medium without FCS (except for conditions when bacteria were added) and S-nitrosoglutathion (GNSO, 100 µM), pure *Salmonella* LPS (50 ng ml^{-1}) (47) or solvent was added. After incubation (37°C, 5% CO_2) for the indicated times, the reagents were removed, and fresh GSNO, LPS, or solvent were added, followed after 10 min by bacterial suspension of *C. jejuni* ($2x10^7$ ml^{-1}) in DMEM.

When heat-inactivated *C. jejuni* were used a 100-fold higher bacterial dose was applied. After incubation (5 h, 37°C, 5% CO_2, unless indicate otherwise) cells were either prepared for measurement of NO, S-protein nitrosylation or transcript analysis, or rinsed and further incubated (4-5 h) with LPS to assess the innate immune response.

RT-qPCR transcript analysis

After rinsing cultured cells twice with DPBS, RNA was isolated with RNA-Bee (Bio-Connect) according to the manufacturer's protocol. Prior to reverse transcription-PCR (RT-PCR), the RNA was treated with 1 µg of DNase (Fermentas) per µg of RNA for 30 min at 37°C. The DNase was inactivated by heating the samples at 65°C for 10 min in the presence of EDTA (2.5 mM, final concentration).

Transcripts of chicken IL-6 and IL-8 (CXCL8-CXCLi2), were determined by real-time RT-PCR using the previously described primers and 5-carboxyfluorescein (FAM) labeled probes (Table 2)(47). RT-qPCR was performed using the Reverse Transcriptase qRT-PCR Master Mix kit (Eurogentec, Seraing, Belgium). Transcript of iNOS, IL-10 and IL-12 were measured in a final volume of 20 µl of one-step RT-qPCR Master Mix plus SYBR Green1 (Eurogentec) using the primers listed in Table 2. Amplification and detection of products were performed using a Roche LightCycler®480 with the following cycle profile: One Reverse Transcription step of 48°C for 30 min, 10 min at 95°C, and 40 cycles of 95°C for 15 sec, and 60°C for 60 sec. For normalization the chicken housekeeping gene glyceraldehydes-3-phosphate dehydrogenize (chGAPDH) was used. For each gene, results were expressed as fold change in mRNA level compared to the non-stimulated cells using the formula: (1)Δ Ct target gene - Δ Ct GAPDH for each sample, (2) Δ Ct target gene treated - Δ Ct target gene control (48). Fold change = $2^{-\Delta(\Delta \text{ Ct gene treated } -\Delta \text{ Ct gene control})}$. Experiments were performed at least for five samples of each condition.

Table 2. Primers and probes used in this study.

chGAPDH	Forward Reverse probe	GCCGTCCTCTCTGGCAAAG TGTAAACCATGTAGTTCAGATCGATGA (FAM)-AGTGGTGGCCATCAATGATCC-(TAMRA)
chIL-8 (CXCL8)	Forward Reverse probe	GCCCTCCTCCGGTTTCAG CGCAGCTCATTCCCCATCT (FAM)-TGCTCTGTCGCAAGGTAGGACGCTG-(TAMRA)
chIL-6	Forward Reverse Probe	GCT CGC CGG CTT CGA GGT AGG TCT GAA AGG CGA ACA G (FAM)AGG AGA AAT ACC TGA CGA AGC TCT CCA-(TAMRA)
chiNOS	Forward Reverse	GGCAGCAGCGTCTCTATGACTTG GACTTTAGGCTGCCCAGGTTG
cIL12	Forward Reverse	GCCAGAAGGAAAACTACTGTCCAT CAATGACCTCCAGGAACATCTCA
cIL-10	Forward Revers	GCTGCGCTTCTACACAGATGAG GCCCATGCTCTGATGA
chG6PH	Forward Reverse	CGGGAACCAAATGCACTTCGT CGCTGCCGTAGAGGTATGGGA

Live imaging of intracellular nitric oxide

To visualize nitric oxide inside NCSU cells, the FL2E trappable fluorescent NO probe (Strem Chemical USA) was used. Hereto, $8x10^3$ cells were seeded onto chambered glass slides (Coverglass system, Lab-TekII, Germany) and grown overnight in DMEM supplemented with 5% FCS at 37^{o}C and 5% CO_2. Four hours after stimulation of the cells with LPS (50 ng/ml^{-1}), the medium was replaced with medium containing freshly prepared FL2E copper complex. After 45 min of incubation in the dark, the cells were rinsed 3x1 ml of DPBS, placed in Hepes buffered saline supplemented with 5 mM D-glucose, and imaged using a NIKON A1R confocal microscope. Fluorescence intensity was calculated using imageJ software. At least five exactly equal areas in every microscopic field were selected and the average fluorescence of the selected areas determined. Fluorescence values from five selected background areas in the same field were subtracted to correct for non-specific fluorescence. The corrected total fluorescence for each condition was measured using formula: CTCF = Integrated Density – (Area of selected cell * Mean fluorescence of background readings**)** (49).

The active copper complex for the FL2E probe was prepared according to the instructions of the manufacturer. Briefly, FL2E was dissolved in DMSO as a 1 mM stock FL2E solution and stored at -80^{o}C. Cupper (II) chloride was dissolved as a 1 mM stock CU solution in pure H_2O and stored at room temperature. Immediately before adding to cells a Cu-FL2E solution was prepared (20^{o}C, portected from light) by adding FL2E solution to an equal volume of copper (II) solution (1:1). The probe solution was diluted in 1 ml of DMEM to a final concentration of 1 μM and added to the cells protected from light.

Statistical analysis

Statistical analysis on all RT-qPCR results was performed on log transformed data using the GraphPad Prism 6.05 multiple *t* test corrected for multiple comparison using the Holm-Šidák method, with alpha=5.000%.

RESULTS

Nitric oxide dampens the chicken TLR4 response

To assess the possible immunomodulatory effect of NO on TLR function in chicken cells, we first determined its effect on inflammatory gene transcription. NCSU macrophages were incubated in the absence and presence of the NO donor *S*-nitrosogluthathione (GSNO) and then stimulated with a low dose of LPS to activate the innate TLR4-MD2 receptor pathway. Quantitative real-time transcript analyses (RT-qPCR) showed that sole exposure to LPS (50 ng ml^{-1}, 5 h) caused a strong increase in IL-6 (400-fold), IL-8 (100-fold), and, to a lesser extent, IL-10 (30-fold) transcripts when compared to non-stimulated cells (Fig. 1A). Transcript levels of IL-12 barely changed under the conditions used (Fig. 1A). Exposure of the cells to GSNO (100 μM, 10 min) prior to stimulation with LPS strongly inhibited the LPS-induced transcriptional response, suggesting a dampening effect of NO on the innate TLR response. A similar effect of GSNO was observed when cells were stimulated with viable *C. jejuni* (2x10^{7} CFU ml^{-1}) instead of pure LPS, although the absolute transcript levels were lower than after stimulation with LPS (Fig. 1B). Exposure of the cells to GSNO alone did not alter gene transcript levels (Fig. 1A and B). Cell viability assays using Alamar Blue staining demonstrated that the used concentration of GSNO (100 μM) did cause cytotoxicity neither at 10 min nor at 5 h of exposure.

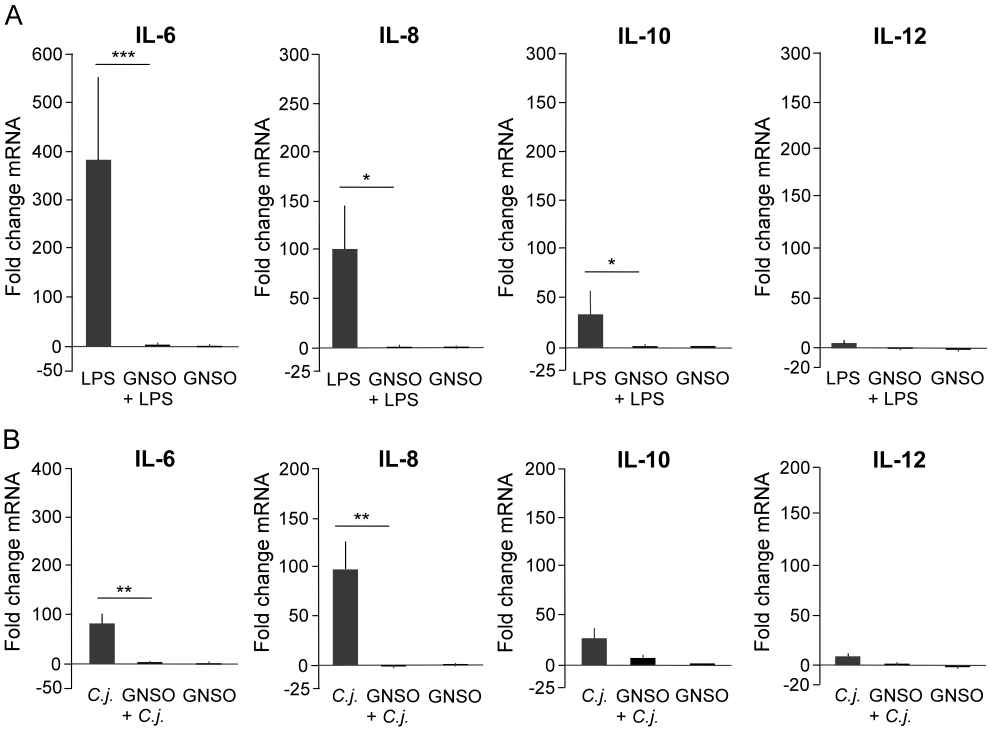


Figure 1. Inhibition of LPS-induced cytokine expression by nitric oxide. Chicken macrophages (NCSU) were stimulated (5 h) with (A) LPS (50 ng ml^{-1}) or B (*C. jejuni* strain 81116) in the absence and presence of GNSO (100 µM) added at t = -10 min. Transcript levels for the indicated inflammatory genes were determined after 5 h of incubation. Data are expressed as fold change in mRNA compared to untreated cells and represent the mean ± SEM of at least four experiments performed in duplicate. Horizontals bars with asterisks indicate statistical significant differences. ***P<0.005; **P<0.001; *P<0.05.

Nitric oxide alters the S-nitrosylation of chicken proteins

One major mechanism by which NO may exert its immunomodulatory effect is via the S-nitrosylation of proteins. This involves the non-enzymatic transfer of NO to cysteine residues on host proteins (13). To investigate whether this mechanism may contribute to the observed dampening of the TLR4 response, we analyzed S-nitrosylation of proteins in chicken macrophages before and after exposure to GNSO. S-nitrosylation was determined by SDS-PAGE after labeling and affinity isolation of S-nitrosylated proteins using the

biotin-switch method (46). Comparison of the protein profiles revealed the appearance of S-nitrosylated proteins after exposure (10 min, 37°C) of the cells to 100-200 μM of GSNO (Fig. 2). These protein bands were not observed when ascorbate was replaced by NaCl during the S-nitrosylation assay, confirming the specificity of the reaction. Interestingly, when the cells were exposed to GNSO in the presence of *C. jejuni*, a reduction in S-nitrosylation seemed to occur (Fig. 2; GNSO+*C.j.*), suggesting that this bacterium is capable to inhibit the S-nitrosylation of proteins in chicken cells.

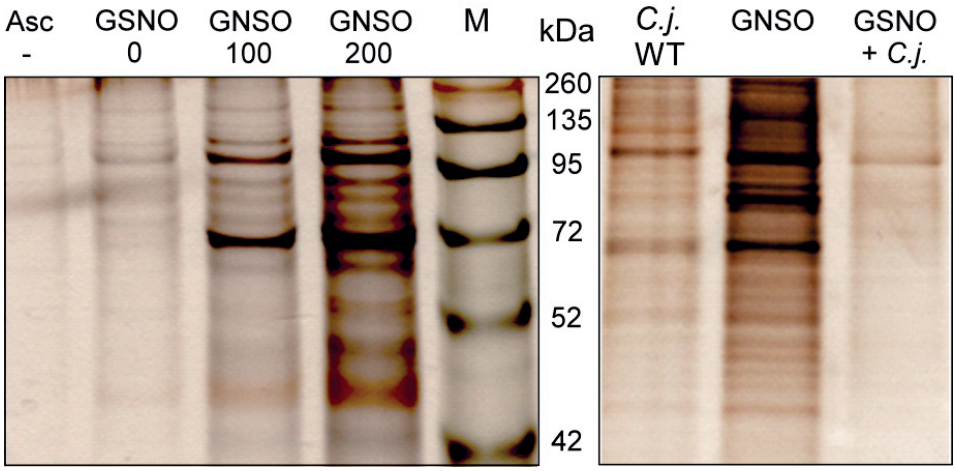


Figure 2. Nitric oxide induced S-nitrosylation of proteins. Chicken macrophages (HD11 cells) were exposed (10 min) to various concentrations of the NO-donor GNSO (100 μM) in the absence and presence of *C. jejuni* strain 81116 (*C.j.* WT) (5 h) or without sodium ascorbate (Asc -; control). S-nitrosylated proteins were biotinylated using the biotin-switch method, isolated using affinity chromatography, separated by SDS-PAGE, and visualized by staining with silver. Equal amounts of total cellular protein were loaded. Results are representative of four independent experiments. M - Molecular weight marker. Molecule mass is indicated in kilodaltons (kDa).

Effect of C. jejuni on NO production in chicken macrophages

The apparent inhibitory effect of *C. jejuni* of the S-nitrosylation was investigated more deeply by measuring the effect of *C. jejuni* on the endogenous cellular NO level which is a major regulator of the S-nitrosylation process. Hereto, chicken macrophages were stimulated (24 h) with LPS in the absence and presence of *C. jejuni* strain 81116 and NO levels were determined in the culture supernatant. As in the presence of oxygen NO is

instantly converted into nitrite, we used nitrite levels as determined by the Griess assay as indicator of NO production. Stimulation of the cells with different concentrations of LPS resulted in the release of large amounts of NO into the medium (Fig. 3). In the additional presence of *C. jejuni* much lower levels of NO (25-90% reduction) were detected (Fig. 3). The lower NO levels may result in a reduction in S-nitrosylation.

In search for the basis of the lower NO levels in the presence of *C. jejuni*, we performed transcript analysis on mRNA isolated from infected and non-infected cells. Quantitative real-time RT-PCR (RT-qPCR) using primers specific for the chicken homolog (iNOS) of the mammalian nitric oxide synthase gene (Nos2) revealed strong (60-150 fold) induction of iNOS transcription in LPS-stimulated as well as *C. jejuni* infected cells (Fig. 3B). This indicates that the observed inhibition of the LPS-response by *C. jejuni* (Fig. 3A) is likely caused by a post-transcriptional mechanism.

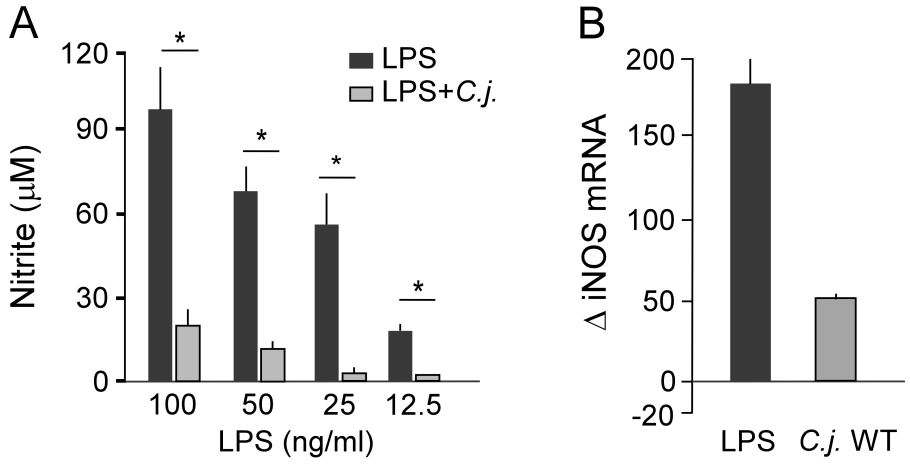


Figure 3. *C. jejuni* reduces LPS-induced NO levels in the medium. Chicken macrophages (12-well plate) were stimulated with the indicated concentrations of LPS in the absence and presence of *C. jejuni* strain 81116. After 5 h, nitrite levels in the culture supernatant were measured using the Griess assay (A) and macrophage iNOS transcript levels were determined by RT-qPCR (B). Results are expressed as absolute nitrite values (Griess assay) or as fold change in mRNA level compared to unstimulated cells (PCR). Results are the mean ± SEM of 5 experiments performed in duplicate. Horizontals bars with asterisks indicate statistical significant differences. ***P<0.0001; **P<0.005; *P<0.05.

***Consumption of exogenous nitric oxide by* C. jejuni**

Possible explanations for the lower NO levels in the medium of LPS-stimulated cells when *C. jejuni* is present are a shortage of the NO precursor L-arginine or the consumption of the produced NO by live *C. jejuni*. As *C. jejuni* barely utilizes L-arginine from the culture medium (51), we tested the ability of *C. jejuni* strain 81116 to utilize NO under the conditions employed. We first determined the consumption of NO using GNSO as a donor in the absence of eukaryotic cells. Toxicity assays using a range of GSNO concentration showed that levels below 100 μM did not reduce bacterial viability. Monitoring of NO levels after the addition of GNSO to *C. jejuni* kept in tissue culture medium (DMEM+5%FCS) showed a strong decline in NO levels within 6 h of incubation. This consumption of NO was not observed in the absence of bacteria (Fig. 4A).

Similar experiments in the presence of eukaryotic cells yielded comparable results (Fig. 4B). To ensure that under these conditions the consumption of NO by *C. jejuni* was being measured, we repeated the experiment with mutants with defects in the enzymes nitrite reductase (81116ΔNrfA) and nitrate reductase (81116ΔNapA) that may play a role in NO assimilation (40). In the presence of the mutants NO levels in the medium remained constant during the entire incubation period of 24 h in contrast to the disappearance of NO observed for the parent strain (Fig. 4B). This strongly suggested that *C. jejuni* rather than the eukaryotic cells consumed the NO and that the periplasmic respiratory reductases NrfA and NapA are required for this process.

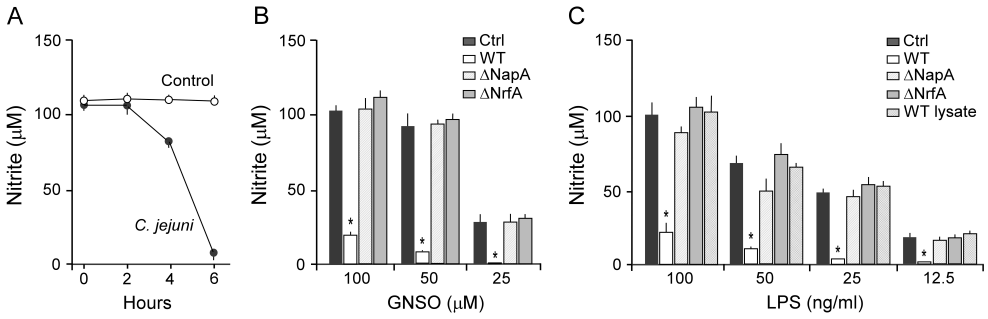


Figure 4. Consumption of nitric oxide by *C. jejuni*. (A) Time course showing medium NO levels during incubation of GSNO in DMEM+5%FCS in the absence and presence of *C. jejuni* strain 81116. (B-C) NCSU chicken macrophages were exposed to (B) GNSO (5 h) or (C) stimulated with the indicated concentrations of LPS (17 h) and thereafter incubated in the absence or presence of *C. jejuni* strain 81116 wild type (WT, $2x10^7$ CFU ml^{-1}), its NapA and NrfA-defective derivatives, or a heat-inactivated bacterial lysate (WT lysate). Nitric oxide levels in the culture medium were determined by the Griess assay at 5 h of *C. jejuni* incubation. Results are the mean ± SEM of five experiments. Asterisks above bars indicate statistical significant differences compared to control. *P<0.001.

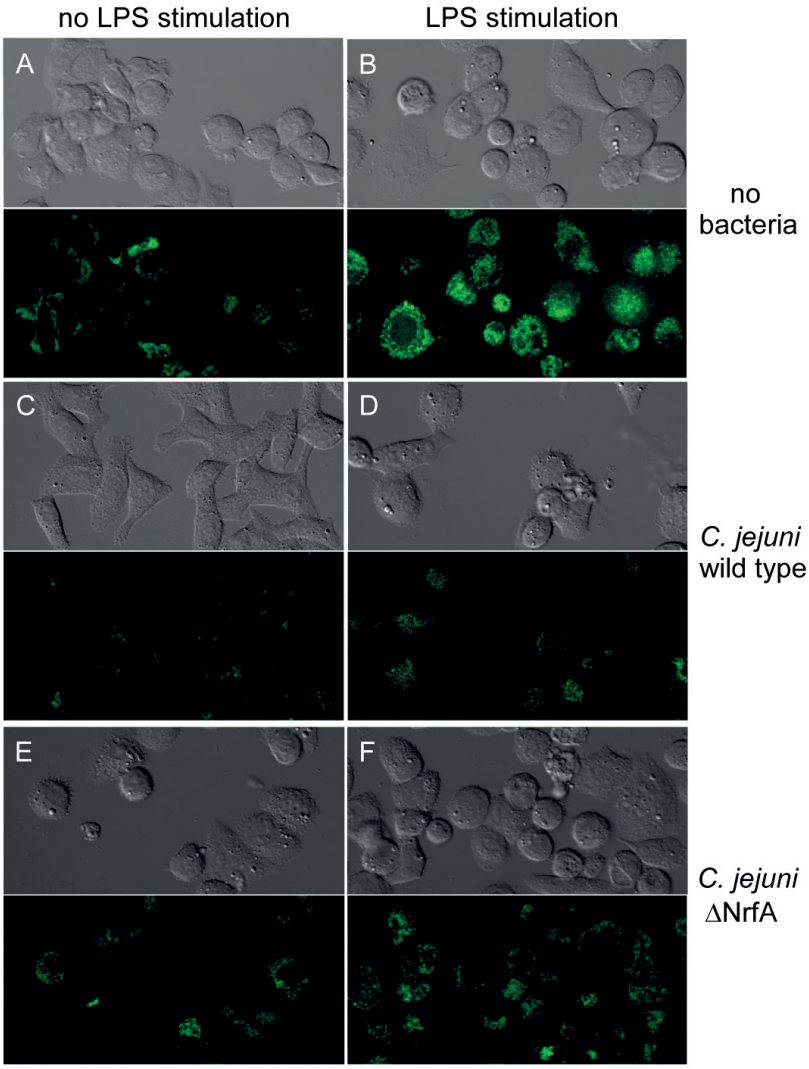
Utilization of host cell-derived NO by C. jejuni

To determine that not only exogenous GNSO but also host cell-derived NO is consumed by *C. jejuni*, macrophages were stimulated (17 h) with LPS to induce NO production and subsequently incubated with *C. jejuni* ($2x10^7$ ml^{-1}) for up to 5 h in a microaerobic chamber (5% O_2, 10% CO_2, 85% N_2). Aliquots of culture supernatants were collected at different time points to determine the NO concentrations. The experiments revealed significantly reduced levels of NO in the culture medium when wildtype *C. jejuni* was present (Fig. 4C). The decline in NO concentration was not observed for the NrfA or the NapA mutants (Fig. 4C). Similar stable NO levels were obtained after the addition of a 500-fold excess of heat-inactivated lysate of *C. jejuni* (10^9 CFU ml^{-1}) (Fig. 4C).

As the Griess assay measures nitrite rather than NO, we further underpinned the hypothesis that viable *C. jejuni* can act as an NO scavenger by determining intracellular NO levels using a trappable fluorescent NO probe (FL2E). This method is based on the direct interaction of cupper with nitric oxide in contrast to the indirect NO measurement by the Griess assay (52). Live imaging of NO in NCSU cells using confocal microscopy showed a strong increase in NO-signal after 4 h of exposure of the cells to LPS (Fig. 5A-B).

Importantly, in presence of LPS together with live *C. jejuni* the NO-signal was strongly reduced (Fig. 5C-D). This reduction in NO was not observed for cells incubated with LPS in combination with the NrfA mutant that was shown to be unable to utilize NO (Fig. 5E-F). Quantification of the fluoresence intensity confirmed the strong induction of NO synthesis in the LPS-exposed cells and the >90% reduction in signal when wildtype *C. jejuni* were present (Fig. 5G).

Figure 5. Localization and quantification of intracellular NO levels as determined by confocal microscopy using the fluorescent NO probe FL2E. Chicken NSCU macrophages were incubated (5 h) in the absence and presence of LPS (50 ng ml^{-1}). Then, *C. jejuni* strain 81116 WT, or the NrfA mutant strain were added and incubated for an additional 4 h. At the end of the 4 h period, the medium was removed and the trappable fluorescent NO probe FL2E was added for 1 h. After removal of the probe, intracellular NO was directly visualized in a Nikon A1R confocal microscope. Fluorescent signal intensity was determined using ImageJ software. The horizontal bar with asterisks indicate that the NO scavenging by *C. jejuni* WT was statistical significant.*$P<0.001$


no LPS stimulation
LPS stimulation
A
B
no bacteria
C
D
C. jejuni wild type
E
F
C. jejuni ΔNrfA
G
rel. fluoescence (x10³)
400
300
200
100
0
*
Ctrl
LPS
WT
LPS +WT
NrfA
LPS +NrfA

***C. jejuni* reverses the NO-mediated attenuation of the LPS response**

To investigate whether the scavenging of host cell NO by *C. jejuni* and the concomitant reduced S-nitrosylation can reverse the dampening effect of NO on the innate immune response, the following strategy was followed. NCSU macrophages were incubated with GNSO (100 μM, 10 min) to increase host cell NO levels and then incubated (4.5 h) in the presence of wild type *C. jejuni* strain 81116 or its NrfA- and NapA-defective derivatives to allow scavenging of the NO and de-S-nitrosylation of proteins. As final step and after removal of the bacteria, LPS was added to assess the effect of the preincubation on the transcriptional cytokine response of the cells. This showed that *C. jejuni* was also able to restore the LPS inflammatory response that was inhibited by pre-treatment of the cells with GNSO (Fig. 6). Under the same conditions, cells incubated with 81116ΔNrfA which is incompetent to scavenge NO, showed no sign of restoration of LPS responsiveness and yielded similar low transcriptional cytokine responses as LPS-stimulated cells exposed to GSNO (Fig. 6). The reversal by *C. jejuni* of the GNSO dampening of the TLR4 response was found for all tested inflammatory gene transcripts (Fig. 6) but was only observed when *C. jejuni* was present in the assay for at least 4-5 h. Stimulation of the cells with LPS within this (4-5 hour) period needed for wild type *C. jejuni* to consume the NO resulted in poor LPS responsenivess of the cells (data not shown). This suggests that a strong reduction of NO levels by *C. jejuni* is needed to reverse the inhibitory effect on the inflammatory cytokine response. Together, our results indicate that NO scavenging by *C. jejuni* dampens the chicken innate immune response.

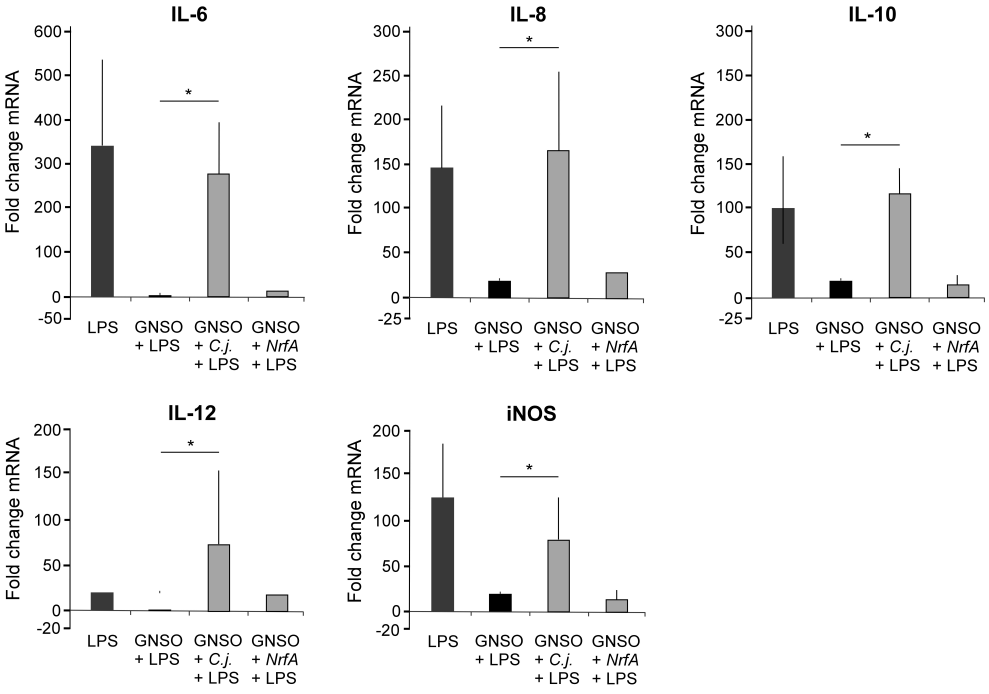


Figure 6. Protective effect of *C. jejuni* on the GNSO-induced inhibtion of the LPS response. Chicken macrophages were stepwise incubated with GNSO (100 µM, 10 min), with either buffer, *C. jejuni* WT, or the NrfA mutant strain ($2x10^7$ CFU/ml, 5 h), and then with LPS (50 ng/ml, 5 h) to stimulate the TLR4-MD2 innate immune receptor pathway. Transcription of the indicated genes was determined by RT-qPCR. Data are expressed as fold change in mRNA compared to untreated cells and are the mean ± SEM of at least four experiments performed in duplicate. Horizontals bars with asterisks indicate statistical significant differences. *P<0.005.

DISCUSSION

Awareness is growing that metabolic interplay between bacteria and eukaryotic cells can tip the balance between bacterial pathogens and the host defense. Here we provide evidence that the principal human foodborne pathogen *C. jejuni* can scavenge host cell-derived nitric oxide and that this results in a change in the S-nitrosylation of host proteins and a modulation of TLR innate immune response. Our results support the concept that bacterial metabolism can actively regulate the host defense and thus should be considered as a determinant of bacterial virulence.

The basis of our work was the observed dampening of the inflammatory gene (IL-6, IL-8) response in cells exposed to NO. This compound strongly reduced the increase in IL-6 and IL-8 transcript levels induced by pure LPS and viable *C. jejuni*. In the absence of NO, LPS not only induced a 100-400 fold increase IL-6 and IL-8 transcripts but also strongly enhanced the transcription of the inducible nitric oxide synthase gene iNOS (Nos2). This was accompanied by the release of large amounts of NO into the medium. Microscopy on cells stained with a fluorescent trappable NO probe confirmed the production of large amounts of NO within the LPS-stimulated cells. The dampening of the TLR response by NO can most likely be attributed to the observed change in S-nitrosylation of host proteins. It is well established that NO levels determine the S-nitrosylation of especially host cell signaling molecules and transcription factors (50, 53) that affect the NF-kB complex that influences inflammatory gene transcription (54).

A major indication that *C. jejuni* may modulate the S-nitrosylation of host proteins by scavenging NO was the discrepancy of a strong induction of iNOS transcript and the low levels of NO in the supernatant by *C. jejuni*-infected macrophages. As this difference between transcript and NO levels was not observed for LPS-stimulated macrophages or after the addition on non-viable *C. jejuni*, we assumed NO scavenging by *C. jejuni* as the causative factor. In many bacterial species, NO is cytotoxic due to inhibition of the bacterial respiration in the electron transport chain (55, 56). In *C. jejuni* the inducible single-domain haemoglobin Cgb can bind and detoxify NO. This process requires upregulation of the Cgb protein in response nitrosative stress (57). *C. jejuni* has also been suggested to be able to apply the constitutively expressed nitric reductase complex NrfA to neutralize NO (35). This protein complex converts nitrite to ammonium, but can also utilitize NO as as substrate (57). Our results indicate that addition of the synthetic NO donor GNSO to a culture of a genetically defined NrfA mutant strain indeed results in a stable high NO levels rather than the NO disappearance observed for wildtype strain. Similar results were obtained when the mutant was used in the infection assays, indicating that also NO produced by activated macrophages is consumed by *C. jejuni* in an NrfA dependent manner. These results indicate the NrfA system of *C. jejuni* as an important element in the scavenging of NO from the environment. Our results resemble to some extent observations in Caco-2 intestinal cells, although in this work the cytoplasmic Cgb protein of *C. jejuni* was suggested to be required for efficient removal of NO from the environment (58).

As NO is a key mediator in cell signaling events, we next hypothesized that the scavenging of NO by *C. jejuni* modulates host cell function. Intracellular NO can react with thiol groups and cysteines in proteins giving rise to the formation of *S*-nitrosylated proteins. *S*-nitrosylation is reversible and has major effect on many proteins including components of the TLR signaling pathway (54). S-nitrosylation has been shown many proteins including MyD88 and the transcription factor p65 that drive NF-kB sensitive cytokine transcription (24). Instrumental in support of the hypothesis was the use of the trappable NO probe that showed a reduction in intracellular NO levels when macrophages were incubated with wildtype *C. jejuni* but not with the NrfA mutant strain. The importance of the use of this NO probe is that it enables direct measurement of NO levels rather than indirect measurement of its oxidation product as is measured by the Griess assay (52). The reduction of intracellular NO levels by *C. jejuni* can be explained by the gaseous nature and readily diffusion of NO across membranes. The scavenging of NO by *C. jejuni* may thus serve as a type of NO sink that depletes host cells from their NO. Analysis of the S-nitrosylation status of the eukaryotic cells in the absence and presence of GSNO using the biotin-switch method, indeed showed strong biotinylation of a multitude of proteins. The effect was not observed in the presence of N-acetylcysteine, which acts as a reductase of the nitrosylation event. Using the biotin-switch method we could demonstrate that wildtype *C. jejuni* but not its NrfA-defective derivative indeed strongly reduced the S-nitrosylation of host proteins. This for the first time indicates that the *C. jejuni* can modulate the S-nitrosylation status of host proteins.

The consequence of the reduced S-nitrosylation was evidenced by the potent TLR innate immune response when the *C. jejuni*-infected macrophages were stimulated with LPS. The strong LPS-induced cytokine response in the presence of the NO consuming *C. jejuni* (but not the NrfA mutant) apparently restored the dampening of the LPS response observed after the addition of GSNO alone (without *C. jejuni*). At first glance, this action of *C. jejuni* may seem awkward as it implies a delay of the dampening effect of NO on cytokine gene expression. Apparently, *C. jejuni* gives preference to detoxify the produced NO and perhaps to utilize it to ensure respiration in the electron transport chain under very low oxygen conditions. At this time, we do not know whether the scavenging of NO by *C. jejuni* fully restores the innate immune response of the host cells or results in a change in

transcript profiles due to a hierarchy in the S-nitrosylation of the relevant involved transcription factors (59).

Together, our results for the first time indicate that *C. jejuni* can scavenge nitric oxide from host cells resulting altered S-nitrosylation and innate immune responsiveness of the host cells. The results underpin the importance of metabolic cross-talk as an important player in the bacteria-host interaction.

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Chapter 6

SUMMARIZING DISCUSSION

Bacteria are an essential part of the gastrointestinal ecosystem of virtually all animal creatures. While most bacteria live in a healthy long-term symbiotic relationship with the host, unwanted guests such as bacterial pathogens may perturb this peaceful world and cause disease. The bacterial pathogen that most frequently colonizes the human gut is *Campylobacter jejuni*. *C. jejuni* is the most common cause of human bacterial foodborne disease in the western world and infects tens of millions of people each year. One major source of *C. jejuni* is contaminated chicken meat. In the chicken gut, *C. jejuni* displays commensal-like behavior and grows to high numbers without significant pathology. Despite the huge human health problems associated with *C. jejuni*, the bacterial and host factors that determine the commensal or pathogenic behavior of *C. jejuni* in different hosts are still largely unknown. Furthermore, attempts to prevent chicken of humans from *C. jejuni* by vaccination have thus far been unsuccessful. The challenging goals of the work presented in this thesis were (i) to pursue the possibility to exploit *in ovo* vaccination technology to immunize chicken with a bacterial subunit vaccine and (ii) to provide fundamental new understanding on the bacterial and host mechanisms that determine *C. jejuni* behavior in the gut in order to develop novel protection strategies.

Immune activation in chicken at the late embryonic stage

Theoretically, a short and effective strategy to reduce the human *Campylobacter* problem should be the *in ovo* immunization of chicken with defined broad protection-inducing *C. jejuni* antigens. This would reduce the *Campylobacter* load in the food chain and reduce the incidence of human campylobacteriosis. However, indications that this seemingly simple solution may actually work is lacking. Successful *in ovo* immunization with bacterial subunit vaccines has barely been demonstrated and doubts exist whether the immune system at the late embryonic stage is sufficiently mature to yield an effective immune response. In **Chapter 2**, we aimed to collect proof-of-principle for the induction of a humoral immune response upon administration of a defined bacterial antigen via *in ovo* immunization technology. In this study, we used purified flagellin (FliC) of *Salmonella* as immunogen. The advantage of *Salmonella* flagellin is that the protein carries intrinsic adjuvant activity through its ability to activate Toll-like receptor 5. *C. jejuni* flagellins lack

the TLR5 binding site and are expected to need the addition of an external adjuvant to elicit a potent immune response.

We delivered the FliC antigen into the amniotic sac of 18-day old fertilized chicken eggs. This location was chosen to stimulate delivery of the antigen via the oral route which may result in more effective mucosal immune responses. Indeed, we could demonstrate an increase in intestinal cytokine and chemokine transcripts shortly after *in ovo* immunization. This effect was likely caused by activation of TLR5 by the flagellin. We showed that TLR5 is expressed by intestinal mucosal cells at the late embryonic stage. Most important, the *in ovo* immunization strategy did mount a FliC-specific humoral immune response. This implies that the mucosal innate immune apparatus and the humoral arm of the adaptive immune system are sufficiently mature at the late embryonic stage to respond to presented antigens. This should provide incentive to the further development of bacterial vaccines that should protect chicken in early life.

Important questions that we did not address in this thesis are the presence of mucosal IgA antibodies after *in ovo* immunization and whether injection with *C. jejuni* flagellins yields similar antibody responses. In separate work published by Radomska *et al*. (1), we tested the vaccine potential of a modified *C. jejuni* flagellin. In these experiments, we used a recombinant NHC flagellin with intrinsic TLR5-stimulating activity. This antigen was constructed by inserting the TLR5 binding site of *Salmonella* FliC into the *C. jejuni* FlaA flagellin (2). This protein is capable to activate TLR5 *in vitro*. Injection of NHC flagellin into 18-day old fertilized chicken eggs resulted in the generation of flagellin-specific antibodies as we showed for *Salmonella* FliC. However, NHC flagellin-specific intestinal IgA antibodies could not be detected and a subsequent *C. jejuni* challenge of the immunized chicken failed to provide protection against intestinal colonization (1). One explanation for the lack of a mucosal IgA response may be the insufficient maturation of the chicken immune system. Such a problem would be difficult to address. An alternative reason for the lack of the intestinal IgA response may be the relatively low vaccine dose that was applied. Immunization with a higher antigen dose or e.g. packaging of the antigen in nanoparticles to better manage antigen release (3) may solve this problem. In the search for protective antibodies directed against *C. jejuni* flagellins, it is important to realize that in the native situation the *C. jejuni* flagellins are heavily decorated with carbohydrates. These sugars may shield the flagellin protein from recognition by antibodies that are generated by immunization with recombinant non-glycosylated flagellin. Thus, vaccination with

glycosylated flagellins rather than recombinant protein derived from *E. coli* may be needed. Additionally, it seems appropriate to search for additional potential cross-reactive *C. jejuni* antigens with vaccine potential. This requires more detailed knowledge of the composition and function of *C. jejuni* antigens in the different hosts.

C. jejuni as opportunistic pathogen in chicken

One of the most intriguing features regarding *C. jejuni* is its difference in pathogenic behavior in the chicken versus the human host. Understanding the underlying reasons is important as it may be instrumental in the identification of novel infection intervention and prevention targets. Numerous factors may contribute to the different *C. jejuni* behavior in humans and chicken. They can be separated into bacterial and host cell factors. On the bacterial side, the local ecosystem of the chicken cecum may reduce the bacterial need to penetrate and damage the intestinal mucosal barrier. Differences in available nutrients, electron donors and acceptors, environmental pH, supportive or competing microbiota, and body temperature may all influence the bacterial phenotype. Host factors that may determine *C. jejuni* virulence include the thickness and composition of the mucus layer, the presence and specificity of mucosal antimicrobial defense, produced host metabolites, the local innate immune status, and the permeability of the intestinal mucosal layer.

In the work described in **Chapter 3**, we addressed the key question whether *C. jejuni* lacks the intrinsic ability to damage the mucosal barrier of chicken or that the chicken host defense limits clinical manifestation of pathology. In order to resolve this issue, we suppressed the immune system of the chicken prior to oral challenge with *C. jejuni*, or, as a comparison, with *S.* Enteritidis. Suppression of the immune system was achieved using glucocorticoids which in mammals are known to prevent an adequate innate and adaptive immune response (4, 5, 6, 7, 8). Our approach clearly showed that *C. jejuni* has the intrinsic ability to pass the mucosal barrier and spread to the liver in immunosuppressed animals. This classifies *C. jejuni* as an opportunistic pathogen in chicken and suggests that, under healthy conditions, the local host defense limits the bacterium to spread from its local niche. Which component(s) of the host defense is (are) responsible for restricting *C. jejuni* to cecum of chicken remains to be investigated. In mammals, glucocorticoids can have very pleomorphic effects. Besides suppression of the immune system, cell metabolism, cell proliferation and differentiation, the salt balance, the function of the central nervous system,

and the reproductive system can be affected (9, 10, 11). We demonstrated that the steroids suppress the TLR responsiveness of chicken macrophages towards bacterial LPS and flagellin, but we cannot exclude the suppression of additional defense mechanisms. We consider a change in the local microbiota as reason for the increased invasiveness of *C. jejuni* in the glucocorticoid-treated birds as less likely as the effect of the drug was already observed at the first day of treatment. A direct effect of glucocorticoids on *C. jejuni* has never been reported.

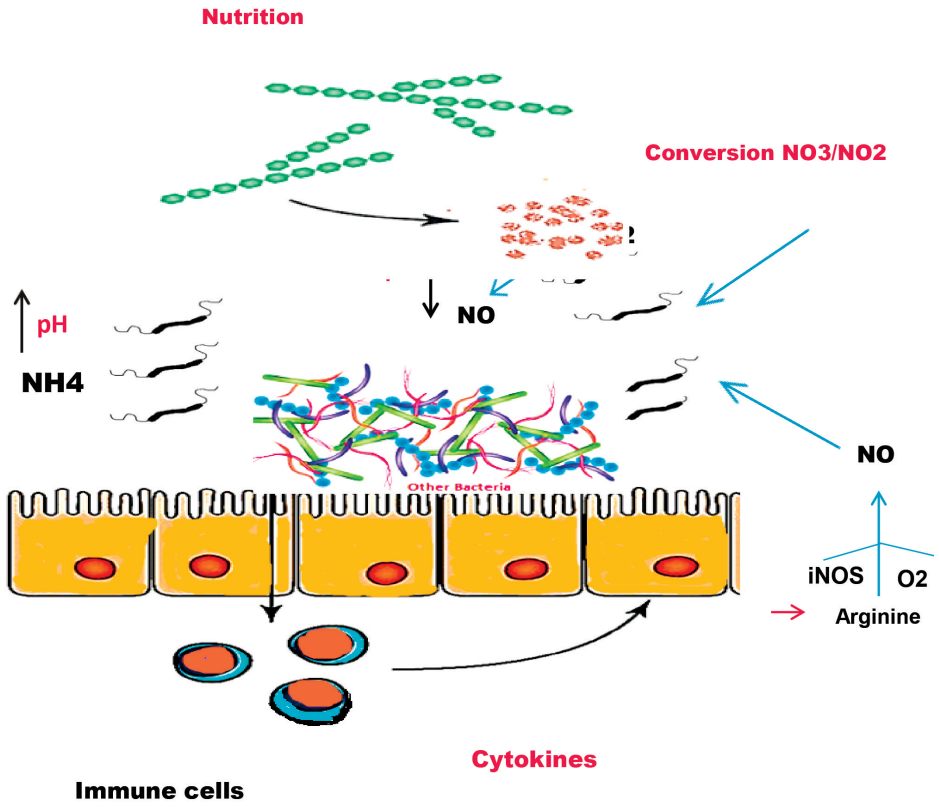

Figure 1. Metabolic interaction between *C. jejuni* and the host mucosa. The interplay between the bacteria and the host may result in the production of metabolites that such as NO or nitrite which may modulate the canonical immune response and the physiological state of *Campylobacter*. *C. jejuni* counteracts the innate host defense by scavenging NO producted by eukaryotic cells.

How relevant are the results obtained with the glucocorticoid-treated chicken for daily poultry practice? Apart from identifying *C. jejuni* as an opportunistic pathogen in chicken, it should be realized that these animals are high susceptible to conditions of acute stress. Stress stimulates the secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland which in turn enhances the production of glucocorticoids by the adrenal glands (12, 13). In poultry farms and especially during the transport of chicken, stress can be expected and this may suppress the function of the host defense. According to our results, this may result in more rapid colonization and rapid systemic dissemination of bacteria like *C. jejuni* and this way perhaps in higher levels of contamination of chicken meat. Thus, control measures to limit bird stress seem not only desirable for sake of animal welfare but perhaps also to limit the risk of human *C. jejuni* infection. In certain regions of the world, administration of very low dose of opium to chickens strongly enhances feather quality and reduces animal stress but this type of stress reduction is probably not a generally feasible and desirable prevention strategy.

***C. jejuni* controls its own micro-environment**

As mentioned above, the colonization and virulence behavior of *C. jejuni* may not only be influenced by host factors but also by local environmental conditions such as available nutrients, available electron donors and acceptors, the local pH, and the composition of the surrounding microbiota. Comparative gene expression profiling of *C. jejuni* isolated from plate cultures and chicken ceca indicated major differences (14, 15) Alterations in gene expression also exist between *C. jejuni* grown at 37^{o}C and 42^{o}C (16) which are the body temperatures of humans and chicken, respectively. These important results indicate that *C. jejuni* has strong adaptive capabilities which may contribute to the different virulence behavior in the chicken and human host. True understanding of the biological significance of these findings awaits the difficult translation of the alterations in gene expression into changes in bacterial phenotype.

The studies described in **Chapter 4** were designed to better understand how *C. jejuni* may adapt to a changing host environment and perhaps influences its own micro-environmental conditions. In contrast to most enterobacteria, *C. jejuni* is a largely non-saccharolytic bacterium that utilizes amino acids as preferred carbon and energy source. Under laboratory conditions, all *C. jejuni* strains that have been tested are capable to utilize L-serine, L-aspartate, L-proline and L-glutamate for growth (17). These amino acids are

also the most abundant amino acids in the chicken ceca. It goes too far to speculate that this correlation points to some type of co-evolution of *C. jejuni* and its chicken niche, but it may explain why *C. jejuni* can colonize the chicken ceca in such massive numbers and without the need of tissue damage to obtain more sufficient food. We discovered how *C. jejuni* exploits its amino acid metabolism to influence the pH of its microenvironment.

During the infection assays described in Chapter 4, we noticed that *C. jejuni* is capable to maintain a neutral environmental pH in contrast to *E. coli* and *S. enterica*. The mechanism beyond this feature was not the absence of the production of acid metabolites as produced by the glucose-utilizing bacteria but rather an active maintenance of a neutral pH by *C. jejuni*. Instrumental in identifying one of the main pH regulating molecules was the finding that L-serine and L-aspartate but not their derivatives pyruvate and fumarate were needed to elevate the low medium pH to neutral values. This pointed to ammonia as regulating factor. To confirm this hypothesis, we developed a novel versatile method to quantify ammonia concentrations in culture media. With use of this method, we could show that *C. jejuni* indeed released large amounts of ammonia into the medium when L-serine or L-aspartate was present. The concentrations of the released ammonia were sufficiently high to elevate the pH of the medium from 4.8 to 7.2. Co-culture experiments in which *C. jejuni*, *E. coli* and *S. enterica* were separated in dialysis tubes, indicated that *C. jejuni* was capable to maintain a local neutral pH in an acid-producing environment.

A most remarkable finding in identifying ammonia as factor that influences the external pH was that the net release of ammonia by *C. jejuni* is inhibited in the presence of glucose (Chapter 4). It is generally accepted that *C. jejuni* cannot metabolize glucose, although certain strains (not investigated here) can utilize L-fucose. What is then the mechanism in *C. jejuni* via which glucose influences the concentration of ammonia in the medium? We cannot answer this intriguing question at this time. In the apparent absence of glucose converting enzymes, it can be speculated that glucose exerts its effect at the post-transcriptional level. The main global post-transcriptional regulator in *C. jejuni* is CsrA (Carbon Storage Regulator A). This RNA binding protein allows bacteria to rapidly modulate a variety of biological processes. In *E. coli* CsrA represses gluconeogenesis and glycogen biosynthesis, and activates glycolysis, acetate metabolism, and flagellum biosynthesis (18). In *C. jejuni,* we and others showed that CsrA regulates the translation initiation of the major bacterial flagellin subunit FlaA (19) and thus affects flagella biosynthesis but whether glucose influences this process has not been investigated. An

alternative mechanism by which glucose may alter *C. jejuni* cell metabolism may be through competition with endogenous substrates for key metabolic enzymes or transferases involved in e.g. the formation of bacterial carbohydrates. *C. jejuni* synthesizes a variety of different carbohydrate structures as building blocks for capsule, LOS, peptidoglycan or the *N*- and *O*-glycosylation machineries. Changes in the central metabolism or the biosynthesis of carbohydrates may cause *C. jejuni* to alter its amino acid metabolism and thus the production, release, or utilization of ammonia. In favor of the competitive binding hypothesis are the relative high levels of glucose that were required to inhibit the net release of ammonia by *C. jejuni*. Overall, it is clear that more metabolic studies (e.g. with use of ^{13}C-glucose) are needed to decipher the regulatory role of glucose in the net release of ammonia.

Although we focused in Chapter 4 on the effect of ammonia on the environmental pH, it is quite possible that it has additional, perhaps even more important effects on the local ecosystem. Maintenance of a neutral external pH is beneficial to most bacteria as it reduces the energy needed to maintain internal pH homeostasis. However, ammonia is also the preferred nitrogen source of e.g. *E. coli* and alterations in external ammonia levels have a major effect on *E. coli* metabolism (20,21). This implies that, theoretically, *C. jejuni* may through the production of metabolites like ammonia, influence the metabolism and growth of other species that are present in the same niche. Conversely, the utilization of glucose by other bacteria may sustain the ammonia release by *C. jejuni* by reducing the inhibitory effect of glucose on this process. Although simplistic, these scenarios stress the importance of considering *C. jejuni* as part of a multispecies community that does not stop its metabolism at its own borders.

Another interesting question regarding *C. jejuni* pathogenesis is whether ammonia influences the health status of the host mucosa. For decades, it is well known that ammonia has major toxic effects on cultured eukaryotic cells (22). The main source of ammonia in cell culture systems is the glutamine catabolism. Dependent on the cell type, toxic effects are already seen at medium ammonia concentrations of 2-3 mM, well beyond the concentrations measured in the *C. jejuni* culture supernatants. On the basis of these findings, the provoking scenario can be imagined that, in the absence of classical virulence determinants, *C. jejuni* may breach the intestinal barrier at least partially by ammonia-induced inhibition of host cell growth i.e. the turnover of the mucosal epithelium. We are currently testing this hypothesis.

Metabolic regulation of the innate immune response by *C. jejuni*

The emerging concept that *C. jejuni* metabolism may actively influence host cell function was investigated with focus on the role of nitric oxide. NO is a key messenger in eukaryotic cells that influences many physiological processes. Intracellular NO regulates cell function through multiple mechanisms including the oxidation of iron-sulfur containing complexes and protein modifications, like ADP-ribosylation and S-nitrosylation. S-nitrosylation is the reversible post-translational modification of cysteine residues that regulates the structure and function of many proteins, especially transcription factors. A well know intracellular target of NO is the p50-p65 NF-κB heterodimer that regulates the transcription of a whole set of inflammatory genes (23, 24). Microbial stimuli such as bacterial LPS and flagellin induce NO production in innate immune cells by upregulation of the inducible enzyme nitric oxide synthase (iNOS). This enzyme catalyzes the conversion of arginine to citrulline and NO. The induction of iNOS by bacterial compounds like LPS and flagellin follows upon their activation of the TLR signaling cascade which ultimately results in nuclear translocation of the NF-κB complex that regulates iNOS gene transcription. Because of the (partial) regulation of the NF-kB signaling pathway by S-nitrosylation, a feedback loop seems to have evolved in which enhanced NO production dampens the TLR response and limits a too strong inflammation.

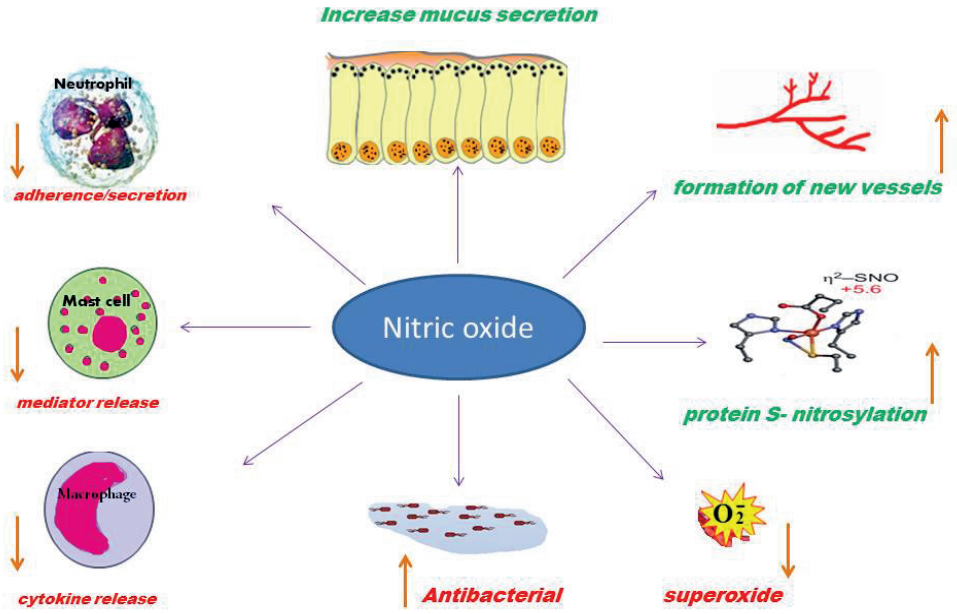

Figure 2. Nitric oxide action in gut. Nitric oxide influences many physiological processes in the gastrointestinal tract. It is an effective vasodilator, an active angiogenesis factor and is involved in stmulating mucus secretion. NO decreases the secretion of neutrophil adherence factors, the release of mediators by mast cells and the release of cytokines by innate immune cells. As a free and active radical, NO reacts with thiol residues in cysteines resulting in protein S-nitrosylation. Nitric oxide contributes to the host cell defense by killing Gram-negative bacteria.

We discovered that host-derived NO has an important role in the cross-talk between *C. jejuni* and chicken macrophages (**Chapter 5**). Addition of the natural NO-donor S-nitrosogluthatione (GNSO) to the macrophages inhibited the LPS activation of TLR4. However, this effect disappeared in the presence of viable *C. jejuni*. We demonstrated that wildtype *C. jejuni* but not an NfrA mutant strain was able to scavenge host-derived NO from the media and the host cells, alleviating the reduction in TLR responsiveness. S-nitrosylation assays for the first time demonstrated that chicken proteins are subject to S-nitrosylation and that this event is influenced by *C. jejuni* NO utilization. Transcript analysis showed that the scavenging of NO by *C. jejuni* resulted in reduced S-nitrosylation

and a restored LPS-induced inflammatory gene expression. The induction of intracellular NO by LPS and its depletion by *C. jejuni* were confirmed *in situ* by the use of a trappable fluorescent NO probe. On the basis of these findings a mechanistic scenario enfolds in which *C. jejuni* targets TLR receptors to induce NO production in host cells and subsequently scavenges the NO to ensure sustained TLR activation and NO production. The utilization of NO by *C. jejuni* may also disrupt the natural regulatory role of NO in the other cellular processes, but this awaits future study.

One chicken-or-egg question that arises from our results is: Does *C. jejuni* deliberately stimulate sustained NO production to derail the innate host defense and gain access to host-derived NO as a nitrogen source, or does the NO scavenging act as a bacterial defense mechanism that protects against NO inhibition of the respiratory electron transport chain? At this time, this question remains unanswered. Sustained NO production may prolong the local inflammatory response but whether this is beneficial for *C. jejuni* is uncertain. However, *C. jejuni* is capable to utilize NO for respiration via reduction by the NrfA complex (25) and to convert it into nitrate presumably via the inducible single-globin domain protein Cgb (26). From this perspective, the sustained production and scavenging of NO may contribute to *C. jejuni* survival, in particular under low oxygen conditions. Considering the central role of NO in host cell biology, it will take considerable effort to unravel whether and how the utilization of host-derived NO by *C. jejuni* influences bacterial pathogenesis and may be used as a target of infection prevention.

Future prospects to prevent *C. jejuni* colonization

In search for strategies to combat bacterial pathogens, research worldwide has mainly focused on the identification of critical virulence determinants such as bacterial adhesions and invasions, immune escape mechanisms and the production of toxins. Yet, this logical approach seems at variance with the main bacterial survival strategy of continuous environmental adaptation and phenotype variation. This seems particularly true for *C. jejuni* which seems to lack classical virulence determinants but displays extensive surface variation and adaptation capabilities. In the present work, we started with investigating the potential of applying *in ovo* immunization as a method to protect chicken against bacteria early in life (Chapter 2). This approach was found to hold promise but awaits identification of the appropriate vaccine targets. The major challenge to prevent *C. jejuni* colonization of the intestinal tract is to identify conserved surface-exposed structures that can serve as

vaccine constituents. This will be difficult considering the extensive phenotypic variation. As *C. jejuni* is covered with a sweet coat composed of a polysaccharide capsule, LPS, glycosylated flagella and *N*-glycosylated proteins, glycans structures seem attractive targets but are also subject to variation. In addition, it remains to be seen whether glycans are sufficiently immunogenic in young chicken to evoke a protective immune response is unknown.

A strong complementary combat strategy is to attack the mechanisms that enable the critical bacterial adaptation to the changing host environment. This can be achieved via directed modification of the local ecosystem in the host such as the innate host defense or the competing microbiota, or by targeting the adaptive mechanisms of the bacterium itself. Our results show that the status of the innate host defense is important to prevent rapid colonization and dissemination of *C. jejuni* in the chicken (Chapter 3). It can be imagined that a strengthening of the innate defense reduces *C. jejuni* colonization. A non-conventional way to stimulate the local host defense is to cause more local mucosal damage. This may be needed to elicit a more effective immune response against *C. jejuni*. Perhaps this can be achieved by drugs that limit mucus production or by the use of attenuated *C. jejuni* strains that are capable to transiently induce tissue pathology. However, such strains do not exist and are unlikely to be applied in animals that are part of the food chain. A perhaps better alternative approach is to alter the host microbiota into a less friendly *C. jejuni* environment. At this time, the role of the bacterial ecosystem of the chicken ceca in the colonization of *C. jejuni* is unknown. Our data indicate that there may be intense communication and mutual use of bacterial metabolites among different bacterial species (Chapter 4), indicating some type of symbiotic relationship. Targeted modification of the composition of the microbiota e.g. through a change in available nutrients, may hold promise but requires much more knowledge of the composition and metabolic and adaptive qualities of the gut microbiota.

A third strategy to reduce *C. jejuni* colonization is to limit the adaptive capabilities of the bacterium itself. *C. jejuni* has different mechanisms of environmental adaptation including slipped strand mispairing, two-component signaling systems that respond to changing environmental cues, and, as we show in Chapter 6, metabolic cross-talk with the host cells. The potential of the use of *C. jejuni* strains with limited adaptive capabilities to reduce colonization is evident from the existence and use of attenuated *Salmonella* vaccine strains. *C. jejuni* mutants with defects in metabolic pathways that poorly colonize the

chicken gut have been reported. Future studies will learn whether *C. jejuni* strains defective in the ability to utilize NO (due to defects in Cgb and NrfA) or to maintain a neutral environmental pH show different colonization behavior but still may induce a protective immune response.

Concluding remarks

The present work demonstrates the exceptional qualities of *C. jejuni* to adapt to changing environments either through internal metabolic adaptation, active alteration of the external environment or the modulation of host cell biology. Although we show that *in ovo* immunization may be an attractive option for the delivery of bacterial subunit vaccines, it is evident that much more work is needed to identify protective *C. jejuni* antigens and to induce local mucosal immunity in early life chicken. Our results indicate that alternative options to reduce *C. jejuni* colonization should be considered. Our data provide evidence that strategies that attack bacterial metabolism and environmental adaptation hold promise. The appreciation that *C. jejuni* is an active part of a large microbiota community that through its metabolism actively interacts with the mucosal tissues and immune system of the host opens new perspective to develop innovative solutions to reduce the worldwide problem of *Campylobacter* disease.

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Nederlandse Samenvatting

De Gram-negatieve bacterie *Campylobacter* is een van de meest voorkomende verwekkers van voedselgebonden infecties bij de mens. Campylobacteriosis treft jaarlijks 0.1-1% van de Europese bevolking. De grootste veroorzaker (>90%) van humane *Campylobacter* infecties is *Campylobacter jejuni* (*C. jejuni*). Hoewel verschillende huisdieren met *Campylobacter* kunnen worden gekoloniseerd, wordt de consumptie van kippenvleesproducten algemeen beschouwd als een belangrijke bron van menselijke infectie in de westerse wereld. De inname van slechts 500-800 bacteriën is al voldoende om een acute enterocolitis te veroorzaken. Symptomen van infectie zijn een acute waterige of bloederige diarree, buikpijn, koorts, misselijkheid en hoofdpijn. De infectie duurt meestal 1-3 weken, waarna in de meeste gevallen volledig herstel optreedt. In een klein aantal gevallen ontstaan echter ernstige complicaties.

Ondanks uitgebreide inspanningen bestaat er nog geen effectieve strategie om de incidentie van *C. jejuni* infectie bij de mens te verminderen. Een mogelijkheid is om de aantallen infectieuze bacteriën op kipproducten te verminderen. Hierbij moet worden gedacht aan maatregelen die de consumptie van rauwe kip beperken en aan strenge hygiëne en technische innovaties op pluimveebedrijven die besmetting en de overdracht van dier op dier beperken. Preventie van de kolonisatie van kippen door middel van vaccinatie is een aantrekkelijk alternatief. Onderzoeken gericht op het ontwikkelen van een *Campylobacter* vaccin voor gebruik bij kippen tonen wel wat vooruitgang, maar er is nog veel onderzoek nodig om een goed werkend vaccin op de markt te brengen. Meer kennis over waarom *C. jejuni* zich gedraagt als een commensale bacterie in de kippendarm en over de interactie van *Campylobacter* met het mucosale weefsel is nodig om een belangrijke stap voorwaarts te maken in de ontwikkeling van een *C. jejuni* vaccin.

Het doel van het onderzoek beschreven in dit proefschrift was om een aantal reeds lang bestaande vragen over het gedrag van *C. jejuni* in de gastheer te beantwoorden. Belangrijke vragen waren: (a) Is het kippenimmuunsysteem in het late embryonale stadium voldoende ontwikkeld om vaccinatie met bacteriële eiwit via *in ovo* vaccin technologie mogelijk te maken? (b) Wordt het commensale gedrag van *C. jejuni* in de kip veroorzaakt door een intrinsiek gebrek aan bacteriële virulentie in de kip en / of door de goede kwaliteit van de afweer van de kip? (c) Is *C. jejuni* wellicht in staat zich in het complexe ecosysteem van darm te handhaven door actief een gunstig eigen micro-milieu te vormen? (d) Draagt

de metabolische wisselwerking tussen *C. jejuni* en het immuunsysteem van de gastheer bij aan bacteriële kolonisatie en ziekte? Antwoorden op deze vragen zijn belang voor het vinden van de Achilleshiel van *C. jejuni* bij het koloniseren van kippen en voor het ontwikkelen van een toekomstig vaccin voor gebruik in kippen.

Hoofdstuk 1 van dit proefschrift geeft een overzicht van de belangrijkste kenmerken van *C. jejuni* en de ziekte die het kan veroorzaken. Dit laat zien dat ondanks dat *C. jejuni* grote effecten heeft op de menselijke gezondheid, de bacteriële mechanismen die bijdragen aan kolonisatie van de darm en het ontstaan van ziekte nog steeds een raadsel zijn. Dit gebrek aan kennis belemmert de ontwikkeling van efficiënte strategieën ter bestrijding van *C. jejuni.*

Hoofdstuk 2 richt zich op de mogelijke toepassing van de *in ovo* immunisatie technologie om kippen al op jonge leeftijd tegen pathogenen bacteriën te beschermen. *In ovo* vaccinatie is tot nu toe nauwelijks toegepast voor bacteriële vaccins. Dit komt gedeeltelijk doordat het nog onbekend is of het immuunsysteem in het late embryonale stadium voldoende ontwikkeld is om de gewenste immuunrespons te verschaffen. Om een beter inzicht hierin te verkrijgen hebben we 18-dagen oude bevruchte kippeneieren geïnjecteerd met gezuiverd flagelline-eiwit van *Salmonella* en hebben we de reactie van het aangeboren en adaptieve immuun tot 3 weken na het uitkomen gevolgd. Onze resultaten tonen aan dat de geïnjecteerde materialen de darm van het embryo bereiken en dat dit gepaard gaat met een verhoogde expressie van ontstekingsgenen in het embryonaal darmweefsel. Dit effect was al meetbaar 24 uur na immunisatie. De verhoogde expressie van chIL-6 en chIL-8 (CXCL8-CXCLi2) wijst erop dat het naïeve embryonale darmweefsel (d.w.z. nog niet eerder blootgesteld aan microbiota) in staat is om op het geïnjecteerde eiwit te reageren. Dit effect komt overeen met de via RT-PCR aangetoonde aanwezigheid van chTLR5 in de darm van 19 dagen oude embryo's. Het is bekend dat herkenning van flagelline door ChTLR5 leidt tot een verhoogde expressie van cytokine genen.

De *in ovo* immunisatie met flagelline leidde tevens tot het ontstaan van flagelline-specifieke antilichamen die tot ten minste tot dag 21 na het broeden konden worden gedetecteerd. Dit resultaat wijst erop dat in kippenembryo's geimmuniseerd met gezuiverd eiwit ook een krachtige adaptieve immuunrespons wordt opgewekt. Dit resultaat impliceert dat niet alleen het aangeboren immuunrespons maar ook het adaptieve immuunrespons

voldoende ontwikkeld is in het late embryonale stadium om te reageren op een enkele injectie met gezuiverd antigeen. Deze belangrijke bevinding biedt de mogelijkheid om ook het *Campylobacter* flagelline vaccin dat in ontwikkeling is, via *in ovo* technologie toe te dienen. Dit heeft grote logistieke en financiële voordelen.

In **Hoofdstuk 3** hebben we de vraag onderzocht of kippen niet ziek worden van *C. jejuni* vanwege een gebrek aan bacteriële virulentie of door de aanwezigheid van een sterk afweersysteem in de gastheer**.** Om deze vraag te beantwoorden hebben we de kolonisatie en verspreiding naar de lever van *C. jejuni* in gezonde kippen en in immuno-gecompromitteerde dieren gevolgd in de tijd. Ter vergelijking werden in deze experimenten ook kippen geïnfecteerd met *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis). Verhoogde verspreiding van de bacteriën in de immuno-gecompromitteerde dieren zou impliceren dat het immuunsysteem van de gastheer de infectie kan beperken, maar ook dat blootstelling van de kip aan stressvolle omstandigheden het risico op bacteriële verspreiding van de darm naar andere organen kan verhogen. We hebben de invloed van het immuunsysteem van de kip op de kolonisatie en verspreiding van *C. jejuni* onderzocht door glucocorticoïden (GC) aan de dieren te geven voorafgaand aan de blootstelling met *C. jejuni*. Onze resultaten tonen aan dat GC-behandeling resulteert in een snellere darmkolonisatie en verspreiding van *C. jejuni* naar de lever. Dit effect ging gepaard met een verminderde transcriptie van specifieke ontstekingsgenen in het cecale weefsel van de GC behandelde kippen. Stimulatie van gekweekte macrofagen met verschillende pro-inflammatoire bacteriële stimuli toonde eveneens aan dat GC behandeling leidt tot een suppressie van het aangeboren immuunrespons. Deze resultaten ondersteunen de hypothese dat *C. jejuni* het intrinsieke vermogen heeft om zich in de kip te verspreiden, maar dat de natuurlijke gastheerverdediging de invasie en verspreiding van *C. jejuni* naar andere organen kan beperken. Meer globaal genomen tonen onze resultaten voor het eerst aan dat de verhoogde corticosteroid spiegels zoals ook bij stress van kippen kan worden waargenomen, kunnen leiden tot een verminderd immuunrespons in de darm en een snellere kolonisatie en verspreiding van bacteriën in de kip. Of een liefdevolle (niet-stressvolle) behandeling van de kip leidt tot een verminderde kolonisatie met *C. jejuni* is in onze studie niet onderzocht.

In **Hoofdstuk 4** was de vraag of *C. jejuni* wellicht een specifieke strategie heeft ontwikkeld om zich te kunnen handhaven in het complexe ecosysteem van de darm. We hebben ons hierbij gericht op het mogelijk effect van het metabolisme van de bacterie op de directe omgeving, in het bijzonder op het gebruik van verschillende koolstof- en energiebronnen en de invloed hiervan op de pH van de omgeving. Kennis van de metabole strategieën van *C. jejuni* om het darmecosysteem te manipuleren kan mogelijk nieuwe mogelijkheden bieden om de kolonisatie van deze bacterie te beperken. Onze resultaten tonen aan dat *C. jejuni* in tegenstelling tot *E. coli* of *S.* Enteritidis grote hoeveelheden ammonium uitscheidt in zijn omgeving en in staat is om de pH van zijn omgeving op een neutraal peil te houden en ook te brengen. Dit effect vereist de aanwezigheid van de met voorkeur door de bacterie gebruikte aminozuren L-serine of L-aspartaat. Het effect treedt niet op met hun respectievelijke gedeamineerde producten pyruvaat en fumaraat. De geproduceerde hoeveelheden ammonium zijn voldoende om een lage pH te verhogen tot neutrale waarde. Een zeer opmerkelijke bevinding was dat de afgifte van ammonium door *C. jejuni* en de daaruit volgende verhoging naar een neutrale externe pH niet optreedt in aanwezigheid van glucose. Dit is vooral merkwaardig omdat wordt aangenomen dat *C. jejuni* glucose niet kan metaboliseren. Samengenomen wijzen de huidige resultaten erop dat *C. jejuni* een glucosegevoelig pH-aanpassingssysteem heeft ontwikkeld om de pH van het milieu te beïnvloeden. Het geïdentificeerde mechanisme kan dienen om de verzuring van de microhabitat te voorkomen die wordt veroorzaakt door bacteriën die glucose gebruiken.

In **Hoofdstuk 5** is onderzocht of het metabolisme van *C. jejuni* wellicht ook kan fungeren als als regulator van het aangeboren immuunrespons van de gastheer. In dit onderzoek lag de nadruk op de rol van stikstofoxide (NO) omdat dit product nauwelijks aantoonbaar was in het medium ondanks hoge expressie van het enzym NO synthase. Om deze reden werden de transcriptiespiegels van cytokine- en chemokine genen en de S-nitrosyleringsstatus van de kippenmacrofagen bepaald in af-en aanwezigheid van *C. jejuni*. Tevens werd de mogelijke productie en het verbruik van NO door de bacteriën gemeten. Deze experimenten toonden aan dat de *C. jejuni* door gastheercellen afgegeven NO kan opnemen. Dit gaat gepaard met een verandering in de S-nitrosylering van gastheercel eiwitten en een verandering van de Toll-like receptor (TLR) afhankelijke aangeboren immuunrespons. Het belang van het verbruik van NO door bacteriën op de immuunrespons werd bevestigd door de afwezigheid van het effect bij gebruik van een *C. jejuni* NrfA

mutant die nauwelijks in staat is om NO te gebruiken. Deze bevindingen tonen voor het eerst aan dat *C. jejuni* de S-nitrosylering van gastheereiwitten kan beïnvloeden. Een krachtige TLR aangeboren immuunrespons was waarneembaar als gevolg van de verminderde S-nitrosylering wanneer *C. jejuni* geïnfecteerde macrofagen werden gestimuleerd met LPS. De sterke LPS-geïnduceerde cytokine reactie die gedempt kon worden door de aanwezigheid van de NO donor GSNO kon weer worden hersteld door het toevoegen van NO-consumerende *C. jejuni*, maar niet de NrfA mutant. Deze resultaten wijzen erop dat bacterieel metabolisme de gastheerverdediging actief kan reguleren en het vermogen tot verandering in bacteriële metabole activiteit beschouwd moet worden als een determinant van bacteriële virulentie. Dit mechanisme biedt wellicht ook aanknopingspunten voor het ontwikkelen van nieuwen vormen van anti-microbiële producten.

Conclusie en slotopmerkingen

Het huidige werk demonstreert de uitzonderlijke kwaliteiten van *C. jejuni* om zich door middel van interne metabole adaptatie, actieve verandering van het externe milieu, en modulatie van gastheercel biologie aan te passen aan een veranderende omgeving. Hoewel we aantonen dat *in ovo* immunisatie een aantrekkelijke optie kan zijn voor de toediening van bacteriële eiwitvaccins, is het duidelijk dat veel meer werk nodig is om beschermende *C. jejuni* antigenen te identificeren en lokale mucosale immuniteit bij hele jonge kippen te induceren. Onze resultaten wijzen erop dat alternatieve opties om *C. jejuni* kolonisatie te verminderen in overweging moeten worden genomen. Onze gegevens bewijzen dat strategieën die het bacterieel metabolisme aanpakken en die daardoor het milieu veranderen veel te bieden hebben. De waardering dat *C. jejuni* een actief onderdeel is van een grote microbiële gemeenschap die door middel van zijn metabolisme actief interactie heeft met het mucosale weefsel en het immuunsysteem van de gastheer, biedt nieuw perspectief om innovatieve oplossingen te ontwikkelen om het wereldwijde probleem van campylobacteriosis te verminderen.

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De man die altijd laat kwam

Mijn moeder vertelde me altijd: Ik was jouw 10,5 maand zwanger!

Als sinds het begin kwam ik te laat!

Ik was zes jaar oud toen ik voor het eerst voet zette op een schoolplein. Nog maar drie maanden van het nieuwe schooljaar waren verstreken toen ik meningitis kreeg. Ik werd 40 dagen lang opgenomen in een halfdonkere kamer en de kans om nog dat jaar naar school te gaan kreeg ik niet. Ik was te laat.

Deze ziekte veranderde veel in mij. Sommige mensen spraken de volgende woorden tegen mij: voor je ziekte was je echt intelligent! En nu dan !!? Niet alleen mijn intelligentie had een afname ondervonden; ons gezin bevatte maar één zoon niemand wilde de enige zoon kwijt. Wat als het kind nog een keer door ziekte gegrepen zou worden? Zo gebeurde het dat ik niet meer met iedereen en overal waar ik wilde mocht spelen. Alvorens ik iets wilde eten moest ik daar toestemming voor vragen.

Ik nam afscheid van de wereld van sport en kinderspelletjes en trad binnen in de wondere wereld der boeken. Zo werd ik een twaalfjarig kind met volwassenen boeken in de hand, in die tijd waren kinderboeken het laatste wat je kon vinden bij ons en als ze er al waren had ik er geen toegang tot. Ik creëerde langzamerhand een eigen wereld met de karakters uit mijn boeken. Toen ik zestien of misschien zeventien was vond ik weer mijn toegang tot de sportwereld. Ik speelde weer voetbal, handbal en andere sporten met de kinderen uit de buurt, maar ik was er te laat bij. Er was geen plekje meer voor mij in de sportwereld.

Het was het laatste jaar van de middelbare school toen die revolutie ongewild gebeurde. Ik had net mijn diploma in de zak, klaar om toe te treden tot de universiteit, toen alle universiteiten gingen sluiten. Het duurde vier jaar tot ze weer hun deuren voor de studenten openden. In dat vier jaar vulde ik mijn tijd als coach en baas van een bibliotheek voor kinderen. Ik was weer laat, dit keer vier jaar te laat!

Eenmaal eindelijk van start op de universiteit, duurde het zes jaar voor ik dierengeneeskunde kon afronden. In die tijd was de universiteit zo gebrekkig in faciliteiten

en was er zo een tekort aan goede professoren dat niemand ook maar interesse had in doorstuderen. In plaats van doorgaan met studeren maakte ik de keuze om de redacteur van een maandblad te worden. Een maandblad dat zich inzette voor de problemen van dorpsbewoners. Dit bleek een moeilijk karweitje te zijn. Ook dit liet ik in de verte achter me en ik ging opnieuw op zoek naar iets anders. De volgende twee jaar hield ik me bezig met lesgeven aan eerstejaars geneeskunde studenten. Dit werk bleek voor mij heel vermoeiend te zijn, zodat ik ook dit ver achter me liet en verderging. Tot slot vond ik mijn weg weer terug naar mijn geboortestad. Hier begon ik weer met lesgeven aan studenten. In die tijd hadden een paar Iraanse universiteiten beetje bij beetje het idee van een PhD-programma geïntroduceerd. Dit programma stond toen nog in de kinderschoenen en enkel kandidaten met een maximum leeftijd van 32 en minstens drie jaar werkervaring werden toegelaten. Zo gebeurde het weer dat deze kans voor mij veel te laat in mijn leven kwam. Om te voorkomen dat ik bij nog een andere kans te laat zou arriveren, besloot ik met 34 jaar te gaan trouwen. Alhoewel, in vergelijking met mijn vrienden was ik er weer eens te laat bij.

Tenslotte, maakte ik tien jaar later de keuze om de stap te zetten en door te studeren. Ik had toen nog maar twee jaar de tijd voordat ik volgens de regelementen de kans zou missen om door te studeren. Ik was hier ook weer laat, maar ik was er bij en daar ging het om. Eenmaal voet op Nederlandse bodem gezet hebbende, hoorde ik over de zware tijden voor de Nederlandse universiteiten en onderzoek kansen. Iedereen sprak vol lof over het verleden. De tijden met een overschot aan budget en een tekort aan studenten. Iedereen sprak weer deze befaamde woorden tegen mij: je bent er laat bij je had eerder moeten komen, het jaar 2001 was een geweldig jaar. Ik had dit zelf ook door, ik was het immers gewend de boot te missen. Een jaar lang focuste ik me op farmacie. Niks verliep zoals het zou moeten en ik was op het toppunt van ontevredenheid, tot ik Jos ontmoette. Het duurde weer veel te lang voor ik hem vond, maar ik bleef net zolang tot ik de verloren tijd had goedgemaakt.

Ik ging geestdriftig op zoek naar een project dat iets te maken zou hebben met kippen en bacteriën. Het departement aan de andere kant van de straat wilde en diermodel van prostaatkanker bij honden maken. Mijn interesse had dit project zeker. Als het met goed gevolg zou verlopen zou ik én dieren betrokken hebben bij mijn werk én mensen. Maar wat

als het mislukte? Met alle twijfels in mijn achterhoofd ging ik op interview. De professor aanvaarde mijn sollicitatie en ik moest twee weken wachten tot er weer contact met mij zou worden opgenomen. In de tussentijd had ik ook een aantal andere plekken gemaild. Toen zag ik plotseling tussen al mijn e-mails een voorstel om drie dagen later te komen solliciteren. Het was een voorstel om te werken op het gebied van kippenvaccines. De vermelde naam was mij tot dan toe onbekend: *Jos van Putten*? Was nergens te vinden in mijn e-mails. Benieuwd als ik was, deed ik een zoekopdracht naar zijn naam op Google. Zijn werkverleden en foto kwamen tevoorschijn. Hij was een Nederlander die overal ter wereld er uit zou springen: overduidelijk 100% Nederlands.

Om elf uur op een hete dag in juli spraken we met elkaar af. Het gedrag van de professor was anders dan alle anderen. Het was op het eerste blik al duidelijk dat deze man alles met een professioneel oog bekeek. Op het gezicht van de professor liet een subtiele glimlach zijn sporen na. Door die glimlach bloeide een gevoel van diepe opluchting bij mij op. De afspraak werd gemaakt om een paar dagen later, na overlegd te hebben met zijn collega's, weer van zich te laten horen. Het mocht zo gebeuren dat precies op de dag dat ik te horen kreeg van het onderzoeksproject over prostaatkanker, ik ook een antwoord kreeg van Jos van Putten. Nu was het zo ver, ik moest nee zeggen tegen een van beiden. Moest ik nu mijn tijd besteden aan het werken aan een vaccine of een prostaatmodel? Als ik alles afwoog tegen elkaar leek prostaat veel beter, als het zou werken. Een vaccine maken? En dat ook nog voor een bacterie die ik tot dan toe nog niet eerder had gezien? Mij was niet eens bekend dat deze bacterie een probleem veroorzaakte bij gevogelte.

Mijn hoofd was een wirwar van gedachten geworden. Van beide professoren wist ik vrijwel niks. De gedachte kwam bij me op om de twee met elkaar te vergelijken en met mijn gevoel te kiezen. De beide mannen vertoonden gelijkenissen met elkaar, de een al dan niet wat ouder dan de ander. Jos van Putten gaf me een "oude-bekende" gevoel die ik niet eerder had gehad. Van de vier plaatsen waar ik had gesolliciteerd had ik bij twee plaatsen geaccepteerd. Echter, bij alle plaatsen had ik in eerste instantie al het gevoel dat ik daar niet zou eindigen. Vanaf het eerste ogenblik dat ik in contact kwam met de professor van de kippen-vaccine, ging er een stemmetje in mijn hoofd rond die zei: "dit is waar je thuis hoort". Hierna mailde ik de professor van het prostaatonderzoek om te laten weten dat ik zijn aanbod helaas laat gaan. De man probeerde mij nog over te halen door te zeggen dat

iedereen in het departement waar ik voor koos arts was en ze niks van dierengeneeskunde zouden weten. Desondanks, had ik mijn besluit al genomen en twee maanden later kon ik beginnen. De afspraak was dat ik een aantal zes maanden contract zou krijgen. Indien alles goed verliep zou een verlenging mogelijk zijn. Twee dagen later kreeg ik een vier-jaar contract voor mijn neus geschoven. Het leek alsof de professor wachtte op een verraste reactie of blijdschap van mijn kant. Ik besloot er niet naar te vragen, hoe onlogisch het ook leek dat ik na twee dagen een vier jaar contract aangeboden kreeg. Tevens had ik in die eerste twee dagen niet echt uitzonderlijke kwaliteiten laten zien. Op dat moment wist ik al dat er een andere reden was en dat ik er ooit achter zou komen. Ik herinnerde me al dat tijdens mijn interview de vragen meer gericht waren op Iran en de cultuur dan op wetenschap en onderzoek. Ik kreeg zelfs de vraag of ik dienstplicht had gehad en of ik het erg vond om met vrouwen te werken. Twee jaar later toen ik het verhaal te horen kreeg van de Egyptische student die een jaar in ons departement was, werd alles duidelijk. Als de professor eerder een Iraniër had gezien, dan was al het detective werk niet nodig geweest.

Alles binnen dit departement was anders dan bij farmacie. Waar farmacie jong en druk was, was dit departement meer bestaand uit mensen van middelbare leeftijd. Het gevoel dat het departement een tweede huis was voor iedereen overheerste. Elke lid van de familie was beschermend over de kern van ons “huis”, het lab. Tegelijkertijd was iedereen ook heel oplettend naar mij toe. Wanneer ik heen en weer ging in het laboratorium leek het alsof iedereen even stilstond om naar mij te kijken. Zodra ik het voornemen had om gebruik te maken van een bepaald instrument, was er wel iemand opeens verschenen die ging “helpen”. Toentertijd had ik het gevoel dat ik de gedachte rondging dat ik als een koe was in een kristallenwinkel, die op het punt stond alles te breken. Hierbij moet ik vertellen dat deze koe negen jaar geen enkel gebroken element heeft veroorzaakt. Bij farmacie schonk niemand aandacht wie je was en wat je aan het doen was. Echter, de eerste keer dat ik de OD van een bacterie wilde bepalen, werd mij verteld dat ik beter eerst een nauwkeurige sample kon maken om HI te besparen. Vanaf de allereerste dagen had ik ook het gevoel dat deze plek mijn tweede thuis zou worden. Een dergelijk gevoel bracht zoveel vreugde in mij teweeg dat ik, zoals Carlos altijd zegt: een deel werd van het meubilair van dit departement.

Tot op dat moment had ik maar drie of vier Nederlandse professoren ontmoet. Geen van deze professoren hield zich bezig met laboratoriumwerk. Geen van deze had ik ooit in een

witte laboratoriumjas gezien laat staan bezig met pipetten gebruiken of cellen kweken. Onze professor, hij deed dit allemaal en hoe nauwkeurig! Zijn manier van werken kan je het best vergelijken met de precisie van een huisvrouw. Alles is altijd op zijn plek en de man kent het klappen van de zweep. Ik had weer het gevoel dat ik ook deze plek te laat had ontdekt, ik had hier tien jaar eerder moeten komen.

De professor hield elke week een gezamenlijke vergadering. Ik kon ze altijd praten, maar voor mij was er nog geen vergadering te bezoeken. Een jaar en acht maanden later werd ik op een dag gevraagd om elke vrijdag om drie uur met een werkreport en mijn plannen langs te gaan op het kamertje van de professor. Het was stipt drie uur toen ik er voor de eerste keer heen ging. De deur van het kamertje stond wagenwijd open en de professor was druk in gesprek met Jaap en Marc. Hun gelach vulde de gangen van het departement. In zijn ooghoek zag hij me staan. Als er iets was dat ik onderhand had geleerd was het wel dat Nederlanders je wel zien, maar je moet altijd wachten tot ze helemaal klaar zijn met hun gesprek (later begreep ik dat dit kwam doordat Nederlanders snel in de war raken, alsof ze de draad van het gesprek kwijt raken). Drie of vier keer liep ik langs het kamertje met mijn map in de hand. Jaap was allang vertrokken, maar Marc zat er nog steeds. De klok naderde vijf uur. In die dagen begreep ik nog niks van Nederland, ik dacht dat belangrijke wetenschappelijke onderzoeken in dit soort gesprekjes werden besproken. Daarnaast maakte Marc een bepaalde angst in mij los waardoor ik besloot om op die dag naar huis te gaan. Een week later had ik weer hetzelfde verhaal. Tot kwart voor vier bleef ik rusteloos rondjes lopen op de gang. De vergadering met Marc was nog steeds aan de gang. Toen Marc eindelijk was vertrokken, stapte ik de kamer van de professor in. Ik werd begroet met een woede die ik nooit eerder van hem had meegemaakt. Hij was woedend dat ik vorige week niet langs was geweest. Ik vertelde hem dat zijn gesprek met Marc tot vijf uur had geduurd en dat ik daarom was weggegaan. Hij vertelde mij simpelweg dat ik naar binnen had moeten komen en mijn afspraak had moeten opeisen. “Als je deze week niet langs was gekomen, zou het voor mij betekenen dat je geen interesse had en dan zou ik geen tijd meer voor je vrij maken.” Zei hij. Ik probeerde het tevergeefs uit te leggen door te zeggen dat ik een paar ik voor zijn deur was langsgelopen en dat hij me ook had gezien. Hierop antwoordde hij met:” Hoe had ik dan moeten weten dat je mij nodig had!?”

Vanaf die dag hadden wij elke week een vergadering op vrijdag. Zes of zeven jaar lang gingen we elke week minstens twee uur zitten en praten. In dat twee uur leerde ik meer van wetenschap, Nederlandse tradities, de geschiedenis, de gewoonten en omgangsnormen dan ik ooit ergens anders had kunnen leren. Deze gesprekken leerden mij dat een Iraniër en een Nederlander zo verbonden met elkaar kunnen zijn dat ze niet eens hoeven te spreken om elkaar te begrijpen. Ik ben iemand die niet makkelijk onder de indruk is van iets of iemand. Vele mensen zijn mijn leven gepasseerd en ik was maar weinig malen echt onder de indruk van iemand. Deze keer moet ik accepteren dat ik dit spel had verloren. Wetenschappelijk denken en van iets simpels een meesterlijke conclusie trekken, waren een van de duizenden dingen die ik heb geleerd. Voor deze dingen zal ik altijd dankbaar zijn.

Op sommige punten kreeg de professor het zwaar te verduren. Ik ben niet iemand die uiterst nauwkeurig te werk gaat. Het maken van een presentatie, het schrijven van een stuk tekst met correcte interpunctie en dit soort nauwkeurige werkzaamheden waren nooit mijn ding. Het spijt me professor dat niet al uw inspanningen tot succes leiden. Misschien begon u wel te laat met dergelijke dingen onderwijzen! Geloof me, het had toch niks uitgemaakt. Zo is mijn vrouw ook al 23 jaar. Als ik eens een keer de afwas doe is het eerste wat zij doet alles nalopen en ergens een drupje olie vinden. Zo gebeurt het dat ik de volgende keer tijdens het koken maar een lepel olie achterlaat op het fornuis, opdat ze het makkelijk kan vinden. Als ik toch een preek krijg dan maak ik het liever makkelijk voor haar. Ik twijfel er absoluut niet aan dat gebrek aan aandacht voor de kleine dingen een hindernis kan zijn op de weg naar succes. Echter, dat het succes absoluut stop is voor mij onwerkelijk.

Ik ben verheugd dat ik heb mogen werken met een professor die niet één keer per jaar zijn kamer verliet. Altijd een luisterend oor, altijd oog voor dingen die alles konden veranderen. Als ik met Jaap had moeten werken was het heel anders gelopen. Jaap stond vaak met één been in Japan en met de ander in Peru. Sinds kort was ook Iran toegevoegd aan de lijst. Als ik een vraag aan Jaap zou hebben was Schiphol vast de vergaderingsplaats. In de drukte van Schiphol zou ik alleen maar moeten luisteren en op zoek gaan naar een lach die de hele ruimte vult. De eerste keer dat ik Jaap zag herinnerde hij me aan Gert. Ook Gert kon je vinden door te zoeken naar zijn lach en zijn ongepaste grapjes.

Karin, een modelvoorbeeld van de huidige Nederlandse vrouw. Niet te vergeten natuurlijk, de twee fijne mannen die ons veel te vroeg verlieten. Nooit zal ik de dag

vergeten dat Wim naar mijn kamer kwam en zei:' Mehdi, ik heb kanker'. Ik had geen idee hoe ik moest reageren. Ook iemand die ons gezelschap te vroeg verliet was Geb. Geb was de ongelukkigste van ons allen. Met al zijn energie, begrip en kennis had hij duizend keer meer kunnen bereiken dan hij al had. Mogen zij beiden in vrede rusten.

Veel studenten kwamen en gingen weer verder. Zoveel zijn het er geweest dat ik sommige nam niet eens meer kan herinneren. We hadden Lieneke, Kasia , die net als Martha, Yaro en Carlos kwam en vertrok om vervolgens weer terug te komen. Of Guus en het Chinese meisje dat een voorliefde had voor de Nederlandse taal. Ik wens iedereen succes toe in hun leven.

Bij ons buurdepartement zijn er veel meer geweest die kwamen en weer gingen. Mensen zoals Henk, die niet lijkt op Jos, Jaap of Gert, maar een collectie is van alle drie. Niet te vergeten het voetbal-duo van het departement. Ik zal er in 2018 niet meer zijn, dus ik kan niet meer gokken op wie de wereldcup gaat winnen. Wat ik wel met 100% zekerheid kan voorspellen is dat Martin de pool gaat winnen en dat arme Edwin weer net als het Nederlandse elftal runner-up zal zijn. Ricardo zal weer derde worden, omdat ze hem weer mild beoordelen. Vergeet Hanna alstublieft niet! Ik weet dat mijn broer Albert dit keer zeker niet meer meedoet. Ik heb nooit een broer gehad, des te fijner dat ik er een heb gevonden. Merci, broer! En natuurlijk dank aan alle meiden van FMA voor alle aardigheid en de grapjes en mijn hartelijk exclusief danken aan Richard voor het bijzonder microscopisch helpen!

Niet alleen in een departement in een vreemd land met alle verschillen met mijn eigen land vond ik rust en vrede. Ook bij ons thuis is het zo. Ik ben getrouwd met een vrouw die niet alleen tien jaar jonger is dan ik, maar ook totaal anders denkt en een andere smaak heeft dan ik. Het huwelijk van een conservatieve, traditionele en gevoelige vrouw die veel waarde hecht aan familietradities met iemand wiens gedachten niet ver liggen van het anarchisme, is op zichzelf al ongekend. Wij hebben zelfs geen overeenkomsten als het om smaak en eten gaat. Echter, wij leven al 23 gelukkig samen zonder ooit een serieuze ruzie te hebben gehad. Ik begrijp dat het moeilijk is om te leven met een man die in een hele andere wereld lijkt te leven. Een hoop dingen die voor anderen van belang zijn hebben geen enkele waarde voor hem. Kleuren, eten, feesten, kleren: alleemaal zijn ze totaal niet van belang

voor hem. Soms heb je hem niet eens iets te zeggen! De meeste mensen praten binnen de familie met elkaar en bespreken alle details met elkaar. Te leven met een man die geen oor heeft voor details, ach wat zwaar! Om eerlijk te zijn heb ik de gewoonte niet om te luisteren, zeker niet als iets twee keer wordt herhaald. Desondanks hebben wij het gered. Wij hebben iedereen laten zien dat anders denken en andere waarden en smaken hebben geen voorbode is van gevechten en ruzies. Ik wist dat ik iemand moest trouwen die mij zou remmen, anders was ik jaren geleden misschien al in een dal gestort (of misschien was ik al bij de Mount Everest!). Nu kan ik zeggen dat naast alle dingen die andere hebben, ik een goeie vrouw heb die mij in leven houdt. Deze goeie vrouw heeft tot betere kinderen.

De dag dat wij in Nederland arriveerden was Soraya negen jaar oud en kende het Engelse Alfabet niet eens. Soroush was drieënhalf oud en sprak het Perzisch niet eens goed. Acht jaar later was Soraya de runner-up in een "public speaking" wedstijd in het Engels en één jaar later was ze Cum-Laude geslaagd. Nu is ze student tandheelkunde op weg naar weer Cum-laude slagen in het eerste jaar. Zij heeft bijna al mijn goede kwaliteiten, maar geen van mijn slechte kwaliteiten. Misschien is zij mijn vrouwelijke versie. Echter, net als haar moeder is ze gehecht aan familie, gevoelig en een felle voorstander van de Perzische cultuur en tradities. Als onze emigratie geen voordelen had voor mij, dan kan ik op zijn minst altijd nog zeggen dat jij alles wat je verdient hebt bereikt en zult bereiken. Ik maak me geen enkele zorgen over jouw toekomst. Ook niet over de toekomst van Soroush, die dezelfde eetgewoonten heeft als ik maar gelukkig verder niet teveel lijkt op mij. Ik dacht altijd dat als ik iets zou willen dat ik het dan ook zou bereiken, maar vaak wilde ik het niet. Soroush is hetzelfde. Als hij iets wil dan zal hij het ook bereiken en vaak wil hij het ook. Mijn grootste zwakte is altijd geweest dat ik geen waarde hecht aan competitie. Niet dat ik bang ben voor verlies. Ik hou enkel niet van wedstijden. Soroush is het tegenovergestelde. Hij is passievol als het op competitie aankomt. Iemand die van competitie houdt verliest, maar wint ook. De angst is dat hij ooit zal strijden voor iets dat de competitie niet waard is. Ik weet dat je op een dag alles zal winnen wat je hart begeert en wil, als je wilt en leert. Je moet willen en leren. Jullie grootste waarde was dat zelfs op de dagen dat we niks hadden, jullie geen gemis of gebrek voelden. Wij kwamen uit Iran met 145 kilogram bagage. Denk altijd aan wat je hebt als je het gevoel hebt dat je ergens aan moet denken!

Niet alleen in mijn gezin, maar ook in mijn familie ben ik soms een vreemde. Vier lieve en goede zussen en dan toch een vreemde. In hun gezelschap ben ik altijd een gast, de leegte tussen ons werd nooit opgevuld. Het is mijn fout. Zo ben ik ook met mijn moeder: een goeie zoon, maar veel te vervreemd. Iemand van wie zij niets weten en die ook niks wil weten van hen, maar toch houden wij van hem en houdt hij van ons zonder dat we elkaar begrijpen.

Misschien is het toch ook niet mijn fout. Naast Jos is er nog iemand die mij goed begrijpt en die ook goed begrijp. Wij zijn geen vreemdelingen voor elkaar en in veel gevallen denken we exact hetzelfde, ook al zijn we in twee verschillende werelden opgegroeid en hebben we een leeftijdsverschil van twee generaties. Claudia leerde mij dat mij als een vreemde voelen niet mijn fout is. Ik kan heel ver zijn maar toch heel dichtbij. Ik heb altijd het gevoel dat ik haar sinds mijn jeugd al ken. Bedankt voor alles.

Het grote verschil tussen ons is dat ik nooit een normaal persoon heb willen zijn. Misschien kon ik dat niet en kan ik dat nog steeds niet. Voor mij is alles een experiment, ik hou van experimenteren. Ik heb veel verschillende carrières, steden, vrienden etc... uitgeprobeerd, allemaal voor het plezier van het experimenteren. Voor mij het leven is een experiment, maar het resultaat is onbelangrijk. Het gaat om het experimenteren. Ik beproef mensen net als bij koken volg ik daar geen specifiek recept bij. Zelfs in het laboratorium heb ik zulke excentrieke experimenten uitgevoerd, dat ik maandenlang moet gaan lezen om te begrijpen waarom ik dat heb gedaan. Ik heb geëxperimenteerd met jullie allemaal en ik heb toegestaan dat jullie mij beproeven. Het resultaat is totaal niet van belang. Het is tijd voor mij om een nieuw experiment te starten. Als je hoort dat ik in de politiek of in het bedrijfsleven ben geëindigd, schrik dan niet! Als ik op een dag niets meer om mee te experimenteren heb, zal ik sterven.

Nu zullen jullie wellicht begrijpen waarom geen enkel resultaat mij tot spanning brengt. Zelfs het behalen van een PhD aan de Universiteit van Utrecht was een experiment! En Einde….

Curriculum Vitae

Mohammad Mahdi Vaezirad was born on March 23th 1961 in Birjand, Iran. After finishing high school in 1980 (Shahrestani school Birjand), he studied veterinary medicine at Tehran University. After graduation in August 1986, he worked as a journalist for three years and then became a lecturer at Azad Zahedan University (medical college) for three years (Zahedan, Iran). In April 1992 he was recruited as a member of academic staff at the faculty of Agriculture of Birjand University. He served as the head of the department of Animal Science for nine years and was the secretary of Academic Affairs for 14 months. At the same time he was working as a clinician in the field of prevention, control and treatment of poultry disease. In November 2006, he moved to the Netherlands to start his PhD study in the department of Pharmacoligal Sciences of the Faculty of Science, Utrecht University. He switched to the Faculty of Veterinary Medicine of the same university in 2008 to work at the department of Infectious Disease & Immunology under the supervision of prof. dr. Jos van Putten. As of November 2017 he will be employed again as an assistant professor in faculty of Agriculture, Birjand University (Iran).

List of publications

1- **Vaezirad MM**, Keestra AM, de Zoete MR, Koene MG, Wagenaar JA, van Putten JPM. 2017. Invasive behavior of *Campylobacter jejuni* in immunosuppressed chicken. Virulence. 8:248-60.

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5- Mortaz E, **Vaezirad MM**. Johnson M, Raats D, Nijkamp FP, Folkerts G. 2008. Salmeterol with fluticasone enhances the suppression of IL-8 release and increases the translocation of glucocorticoid receptor by human neutrophils stimulated with cigarette smoke. J Mol Med (Berl). 86:1045-56.

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7- **Vaezirad MM,** Bleumink-Pluym NM, van Putten JPM**.** *Campylobacter jejuni* modulates the innate immune response by altering protein S-nitrosylation. Submitted for publication.

8- **Vaezirad MM,** Koene MG, Wagenaar JA, van Putten JPM. Chicken immune response following in ovo delivery of bacterial Flagellin. Submitted for publication.