New Features of Sialylated Lipo-oligosaccharide Structures in *Campylobacter jejuni*

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New Features of Sialylated Lipo-oligosaccharide Structures in *Campylobacter jejuni*

Nieuwe functies voor *Campylobacter jejuni* gesialyleerde lipo-oligosaccharide structuren

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Is everybody in?... Is everybody in?... Is everybody in
The ceremony is about to begin..
Jim Morrison

CONTENTS

Chapter 1

General introduction aims and outline of this thesis

GENERAL INTRODUCTION

The zoonotic human enteric pathogen *Campylobacter jejuni* is acquired by humans through contaminated water, poultry, shellfish and pets [1]. Motility, chemotaxis, glycosylation and lipo-oligosaccharides (LOS) structures are all different virulence features exploited by *C. jejuni* to adhere, invade, adapt and survive in a mammalian host [2-11]. The most interesting one is the LOS structure, which is phase variable [12, 13]. *C. jejuni* LOS phase variation not only provides host adaptation abilities [14], but also protection against human serum [8]. Next, LOS is an important virulence factor used by *C. jejuni* to invade intestinal epithelial cells [15, 16]. To date, five major and distinct LOS biosynthesis gene clusters, here referred to as LOS classes, have been described for *C. jejuni* [17], and this number is continuously growing [18]. Analysis of the LOS biosynthesis gene loci of complete *C. jejuni* genomes revealed these loci to be highly variable [18, 19]. Although the number of *C. jejuni* LOS classes is continuously growing and its LOS biosynthesis genes found to be highly variable [17, 18], a specific molecule, to be precise, sialic acid, enables us to separate *C. jejuni* into two main groups. One *C. jejuni* group that is able to sialylate their LOS structures (LOS class A, B and C) and ones disabled in sialylation of their LOS structures (LOS class D, E and others). Sialic acid transfer to the LOS structures on *C. jejuni* occurs by two sialyltransferases; a α2,3/α2,8 sialyltransferase named Cst-II [20] and a α2,3 sialyltransferase named Cst-III [19]. Interestingly, the two LOS classes A and B, are strongly associated with the post-infectious complications Guillain-Barré Syndrome (GBS) and Miller Fisher Syndrome (GBS and MFS), respectively [21]. These two classes harbor the *cst*-II gene, until now the only functionally established marker for GBS [21, 22]. Noteworthy, Cst-II and Cst-III mediated sialylation of LOS structures (LOS classes A, B and C) is also associated with severe gastro-enteritis, bloody stools and another post-infectious complication, Reactive Arthritis [23].

A key feature of Cst-II and Cst-III mediated sialylation of LOS structures on *C. jejuni* is that they mimic similar structures, called gangliosides, on the human peripheral nerves [24]. It is thought this molecular mimicry is the most important factor in GBS induction, because in a subset of enteritis patients antibodies are generated that have the ability to cross-react with both *C. jejuni* ganglioside-like LOS and human nerve gangliosides. Binding of these antibodies will lead to removal of the myelin sheet by macrophages, ensuing loss of nerve function and GBS induction [25]. Worth mentioning, approximately 50 - 60% of the *C. jejuni* enteritis isolates are able to express ganglioside mimics [21], but only 1 in 1000 *C. jejuni* infections results in the development of GBS [26]. Although there are strong indications that cross-reactive antibodies are important for the induction of GBS [27], little is known about the mechanism(s) that lead to the development of these cross-reactive antibodies.

An important feature of the LOS classes A and B is that they can be horizontally transferred between *C. jejuni* isolates [28, 29]. Presence or absence of these loci might be regulated by a *C. jejuni* defense system that target mobile DNA. Such a system could be the CRISPR-Cas (Clustered Regulatory Interspaced Short Palindromic Repeats array and associated *cas* genes) sys-

tem, an adaptive immune system of bacteria and archaea that neutralizes mobile DNA [30-32]. In 2005, three independent groups reported that CRISPR-Cas contains small sequences that are 100% identical to bacteriophage or plasmid sequences [33-35]. In 2007 and 2008, it was shown that these sequences called spacers could be acquired *de novo* following bacteriophage or plasmid challenges, respectively, which in turn ensured bacteriophage or plasmid resistance [36]. A structural feature of all CRISPR-Cas systems is the presence of 6 - 20 CRISPR-associated (*cas*) genes located upstream of the repeat sequence locus [37]. The Cas proteins are implicated in the processing of the transcribed CRISPR spacers and cleavage of foreign nucleic acids bound to CRISPR spacers [30, 31].

Bacterial comparative genomics revealed that CRISPR-Cas can be distinguished in diverse bacterial species-specific groups, recently specified in three main CRISPR-Cas subtypes [37]. The Type-II CRISPR-Cas system is the most reduced version of all known CRISPR-Cas systems [37, 38]. Type II CRISPR-Cas is based on the CRISPR-Cas system present in *Neisseria meningitidis* isolate Z2491 [38]. Nearly all bacteria bearing this subtype contain two subtype-specific *cas* genes, *csn1-2*, in addition to the more conserved *cas* genes *cas1-2* [37, 38]. In this subtype the spacer lengths are only about 30 bp [37]. Species belonging to the Type-II CRISPR-Cas are all pathogenic, vertebrate host-associated bacteria, except one; commensal intestinal-inhabiting *Wolinella succinogenes* [37].

An approach, using mathematical models, to tackle CRISPR evolution and population dynamics of CRISPR-encoding bacteria predicted that the circumstances which enable maintenance of the CRISPR-Cas system are narrower when there is cell envelope resistance to bacteriophages [39]. This means that if mutations in a bacterium generate cell envelope-mediated bacteriophage resistance, it could influence CRISPR-Cas perpetuation. Since bioinformatic analyses had revealed that the Type II CRISPR-Cas system was mainly associated with bacterial species able to sialylate their cell envelope [24, 37, 40-42], we addressed whether the reason for a strongly reduced CRISPR-Cas system in *C. jejuni* is sialylated cell envelope mediated bacteriophage resistance in **Chapter 2**.

For *N. meningitidis*, *H. influenzae* and *C. jejuni* it is established that a sialylated cell envelope is important for escape from denditric cells and protection against human serum [9, 43], respectively. These results were generated by knock-out mutagenesis of bacterial species specific sialyltransferases [9, 43]. For *C. jejuni* presence or absence of sialylated LOS was confusingly linked with no effect, decreased or increased intestinal epithelial invasion [8, 15, 16]. Unfortunately, these studies were approached with only a wild type and mutant isolate, but were never set up with a large collection of *C. jejuni* isolates to address whether or not sialylated LOS structures were important for intestinal epithelial invasion. We therefore addressed the importance of sialylated LOS in intestinal epithelial invasion in **Chapter 3**, by using not only a large heterogenic *C. jejuni* collection, but also three sialyltransferase mutant isolates lacking sialylated LOS and a complemented sialyltransferase mutant with restored sialylated LOS expression.

C. jejuni and other bacteria that invade eukaryotic cells often employ common cellular pathways such as endocytosis [44-48]. Endocytosis consists of early and later stages that can be conveniently distinguished using protein markers. The protein markers frequently used to study the different endocytic stages are the early-endosome associated protein 1 (EEA1), the GTPase proteins Rab5 and Rab7 and the lysosomal-associated membrane protein 1 (LAMP-1). EEA1 and Rab5 are involved in the early stages of endocytosis [49], Rab7 marks later endocytosis stages [50], and LAMP-1 marks the end stage, when late endosomes are fused with lysosomes [51, 52]. These protein markers can be visualized by immuno-histochemistry so one can microscopically follow the trafficking process of, e.g. *C. jejuni* from the apical cell surface, across intestinal epithelial cells, to the basolateral cell surface.

In **Chapter 4** we addressed the role of sialylated LOS structures in endocytosis and translocation across intestinal epithelial cells. Studying the sialylated LOS structures in relation to epithelial translocation is of importance, since the Cst-II and Cst-III expressing *C. jejuni* bacteria are linked with severe gastro-enteritis and bloody stools in *C. jejuni* diseased patients [23].

Next to involvement in *C. jejuni* pathogenesis, LOS classes could be useful for typing purposes [17, 18], enabling researchers to separate virulent from less virulent isolates. The epidemiological relevance of *C. jejuni* LOS gene screening could be further fine-tuned by combining results from other molecular-typing tools (e.g., multilocus sequence typing [MLST] [53, 54], pulsed-field gel electrophoresis [PFGE] [55], PCR restriction fragment length polymorphisms [RFLP] [56] and sequencing [57]). These typing methods are commonly used to study epidemiology in poultry farms, currently discussed to be the basis where *C. jejuni* should be eliminated before it can infect humans after processing. In **Chapter 5** we studied whether there was a difference or correlation between genotypic diversity, lipo-oligosaccharide gene locus class variation, and Caco-2 cell invasion potential of *C. jejuni* isolates from chicken meat and humans.

Unfortunate, the *C. jejuni* transmission routes are not well understood, which makes this bacterium an obligatory contaminant in the food chain [58]. Not only has this lack of knowledge made it difficult to eliminate this bacterium from the food chain. This lack of knowledge has led to an excessive use of antibiotics at poultry farms resulting in increased antibiotic resistance of the *C. jejuni* bacterium [59-61]. Plasmids and mobile genetic elements are known distributors of antibiotic resistance genes [62, 63]. A significant proportion of *C. jejuni* isolates harbors plasmids and the contribution of plasmids in the pathogenesis and antimicrobial resistance of *Campylobacter* infections has been studied since the early eighties [64]. Two large plasmids have been isolated from this bacterium, pVir and pTet [65, 66]. The plasmid pVir has been implicated earlier in the virulence of *C. jejuni* [65, 67] and pTet carries tetracycline resistance [65]. In addition, a significant association was reported on the presence of pVir with bloody stools in a Canadian study [67]. Next, data on pVir also suggested its importance in *C. jejuni* virulence [65]. In **Chapter 6** we investigated whether pVir or pTet were associated with bloody stools, the Guillain-Barré or Miller Fisher Syndrome in The Netherlands.

Although *C. jejuni* is the most frequently identified infection preceding GBS, it has been questioned whether or not other *Campylobacter* species, including *C. curvus*, *C. upsaliensis* and *C. coli*, could be similarly involved [68-70]. This is of interest, because it could suggest that the factor(s) involved in *C. jejuni* induced GBS crossed species barriers or that other bacterial factors then sialylation of *C. jejuni* LOS are involved in GBS induction. In **Chapter 7** we presented two GBS patients, who both were infected with *C. coli*. The *C. coli* isolates were further analyzed on whether ganglioside mimic structures or other features were involved in GBS induction in these two patients.

AIMS OF THIS THESIS

The specific aim of this thesis was to improve our insight in the biological features of *C. jejuni* sialylated LOS structures (ganglioside mimics) and plasmids; this to enhance our knowledge on why a subset of patients infected with *C. jejuni* develops GBS. A second aim of this thesis was to identify new GBS markers in *C. jejuni*, since Cst-II mediated ganglioside mimics do not seem to be the single factor involved in GBS induction. A third aim of this thesis was to address the role of *C. coli* in GBS.

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OUTLINE OF THE THESIS

In **Chapter 2** we present data showing that the presence of ganglioside mimic structures on *C. jejuni* isolates play an important role in protection against bacteriophage attacks, which affects a more basic bacteriophage defense system, namely Type II CRISPR-Cas. We observed that the affected bacteriophage defense system Type II CRISPR-Cas in *C. jejuni cst*-II harboring isolates has a crucial role in *C. jejuni* pathogenesis. Next, we reveal that the CRISPR-Cas system harbors DNA polymorphisms associating strongly with the earlier established GBS marker *cst*-II. In addition other DNA polymorphisms in the CRISPR-Cas system were found to be new GBS and enteritis markers.

In **Chapter 3**, we show that ganglioside mimic structures are not only useful for bacteriophage defense, but may also increase the virulence of *C. jejuni* isolates. We observed that human *C. jejuni* isolates that express gan glioside mimic structures invaded intestinal epithelial cells in higher numbers, results that we were able to conform by knock-out mutagenesis and complementation of the sialyltransferase *cst*-II gene needed for sialylation of *C. jejuni* LOS.

Intra-cellular trafficking and translocation is visualized in **Chapter 4**. In this chapter we show that *C. jejuni* ganglioside mimics contribute to enhanced entry into intestinal epithelial cells. Inside the cell, *C. jejuni* was found to use the endosomal pathway for cellular trafficking as was visualized by specific endosomal markers EEA1, Rab5, Rab7 and LAMP-1. A specific endo-lysosomal stain, Lysotracker DND-99, and an intra-cellular survival assay revealed that only a small percentage of *C. jejuni* isolates was able to survive. Although all *C. jejuni* isolates reveal in Caco-2 cells an equal survival percentage, increased endocytosis by ganglioside mimic expressing isolates was in that way linked with increased cellular translocation across intestinal epithelial cells.

PCR screening in **Chapter 5** showed that 87.1% (101/116) of isolates could be assigned to LOS class A, B, C, D, or E. A specific subset of *C. jejuni*, namely the LOS class C expressing isolates, harbors a *cst*-III instead of *cst*-II sialyltransferase. LOS class C could be assigned to the MLST locus CC-21. Another MLST locus, CC-206, was over represented by LOS class B expressing isolates. Next, we revealed that there was no difference between chicken and human isolates in invasion of epithelial cells and showed that ganglioside mimics are an important factor for increased virulence.

The *C. jejuni* plasmid pVir was found to associate with increased virulence and bloody stools in a Canadian study, which we could not corroborate in **Chapter 6**. We show that only a small percentage of the Dutch isolates harbored the pVir plasmid. Another large plasmid pTet could only be correlated to increased resistance against the antibiotic tetracycline and both pVir and pTet could not be linked to GBS.

In **Chapter 7** we present two GBS patients who were both infected with a *Campylobacter coli* strain. A link between *C. coli*, molecular mimicry and GBS remained obscure in this study.

Chapter 2

A distinct link between *Campylobacter jejuni* bacteriophage defense, virulence and Guillain-Barré Syndrome.

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In preparation for Current Biology

Chapter 3

The sialylated lipo-oligosaccharide outer core of *Campylobacter jejuni* is an important determinant for epithelial cell invasion

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ABSTRACT

Campylobacter jejuni is a frequent cause of bacterial gastroenteritis world wide. Lipo-oligosaccharide (LOS) has been identified as an important virulence factor that may play a role in microbial adhesion and invasion. Here we specifically address if LOS sialylation affects the interaction of *C. jejuni* with human epithelial cells. To this aim, 14 Guillain-Barré Syndrome (GBS) and 34 enteritis-associated strains, the 81176 reference strain and 6 Penner serotype strains, were tested for invasion into two epithelial cell lines.

C. jejuni strains expressing sialylated LOS (class A, B and C) invaded significantly more than non-sialylated LOS strains of classes D and E ($p < 0.0001$). To further explore this observation, we inactivated the LOS sialyltransferase (Cst-II) via knock-out mutagenesis in three GBS-associated

C. jejuni strains expressing sialylated LOS (GB2, GB11 and GB19). All knock-out strains displayed significantly reduced invasion compared to the respective wild types. Complementation of a Δ*cst*-II mutant strain restored LOS sialylation and reset the invasiveness to wild type levels. Finally, formalin-fixed wild type strains GB2, GB11 and GB19, but not the isogenic Δ*cst*-II mutants that lack sialic acid, were able to inhibit epithelial invasion of viable GB2, GB11 and GB19 strains. We conclude that sialylation of the LOS outer core significantly contributes to *C. jejuni* epithelial invasion and may thus play a role in subsequent post-infectious pathologies.

INTRODUCTION

Campylobacter jejuni is recognized as a leading cause of bacterial gastroenteritis worldwide. Poorly handled or improperly cooked poultry meat, raw milk, pets, and untreated water are thought to be sources of infection [1]. The disease spectrum caused by *C. jejuni* ranges from asymptomatic infection to severe inflammatory bloody diarrhea [2]. Furthermore, *C. jejuni* infection has been associated with the development of post-infectious complications such as the Guillain-Barré syndrome (GBS) [3]. The apparent variation in gastrointestinal disease outcome is likely to be affected by the expression of virulence factors that are associated with specific pathogenic mechanisms, e.g., *C. jejuni* motility [4], attachment [5], and invasion [6-8]. Motility and chemotaxis appear to be necessary for the epithelial adherence of *C. jejuni*, whereas the expression of functional flagella may determine the capacities of *C. jejuni* to invade the epithelium and to effectively colonize the mouse intestine [8-11].

Next to the role of flagella in the regulation of *C. jejuni* invasiveness, lipo-oligosaccharide (LOS) structures have generally been implicated in microbial invasion [12-18]. To date, five major and distinctive LOS biosynthesis gene clusters, referred to here as LOS classes, have been described for *C. jejuni* [19], and this number continues to increase [20]. Sequencing and microarray analysis of the LOS biosynthesis gene locus of the *C. jejuni* genome have also revealed this locus to be highly variable [15, 21], which may contribute to the variation in *C. jejuni*-associated pathologies. Furthermore, it has been shown that *C. jejuni* strains may also acquire these LOS synthesis genes from other *C. jejuni* strains by means of horizontal exchange [22-23].

A subgroup of *C. jejuni* strains that express the LOS class A, B, or C gene locus harbor genes involved in sialic acid biosynthesis and are therefore able to synthesize sialylated LOS [21, 24-26]. The *cst*-II gene encodes a sialyltransferase [27] that is necessary for the transfer of sialic acid onto the LOS core in *C. jejuni* class A and B strains. *C. jejuni* class C strains depend on the cst-III gene for LOS sialylation. Hence, only *C. jejuni* strains expressing LOS class A, B, or C are capable of LOS sialylation. Previously, we have shown that the presence and expression of the cst-II gene is specifically associated with GBS and is required for the induction of anti-ganglioside antibody responses, which are the hallmark of this post-infectious complication [25, 28]. Based on this prior work, we hypothesized that LOS sialylation (and consequently *C. jejuni* LOS subclasses) may be involved in *C. jejuni* invasiveness.

Therefore, a panel of 48 human isolates and 7 human control strains were assessed for invasiveness into two human epithelial carcinoma cell lines (Caco-2 and T84). To specifically explore the role of sialylation, we generated three GBS-associated sialyltransferase (Cst-II) knockout *C. jejuni* strains (GB2Δ*cst*-II, GB11Δ*cst*-II, and GB19Δ*cst*-II). These GB2Δ*cst*-II, GB11Δ*cst*-II, and GB19Δ*cst*-II mutants were tested for their abilities to adhere to and invade Caco-2 cells. Finally, we investigated whether complementation of the Δ*cst*-II mutant would restore the invasion-associated function of this gene product.

RESULTS

LOS sialylation is associated with increased epithelial cell invasion. LOS sialylation is associated with increased epithelial cell invasion. We observed a wide range of invasion capacities among the *C. jejuni* strains (**Supplemental Table 1**). Categorization of *C. jejuni* strains into those carrying sialylated (n = 30) and non-sialylated (n = 18) LOS established that the sialylated-LOS producers, classes A, B, and C, were more invasive than the non-sialylated-LOS producers, classes D and E (median CFU per millilitre, 408,300 for classes A, B, and C and 11,190 for classes D and E; $p < 0.0001$) (**Fig. 1A**). Notably, on average, the GBS-associated strains (n = 14) invaded significantly better than the enteritis-associated strains (n = 34) (median CFU per millilitre, 632,700 versus 49,630, respectively; $p = 0.0046$) (**Fig. 1B**).

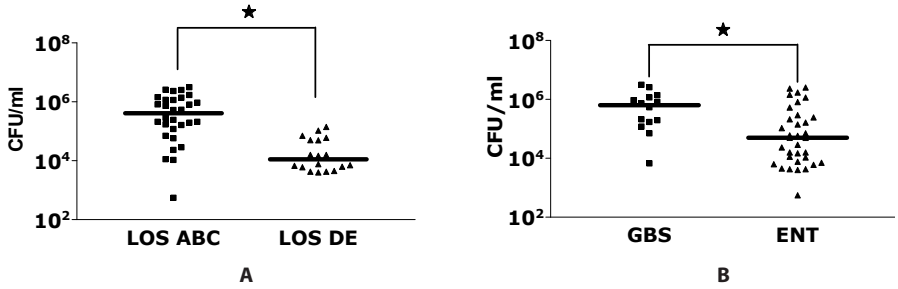

Figure 1 The invasiveness of *C. jejuni* is dependent on sialylation of the LOS. Scattergrams show the invasion of Caco-2 cells by Dutch *C. jejuni* strains, categorized with respect to the type of LOS that is expressed (sialylated LOS of classes A, B, and C [n = 30] versus non-sialylated LOS of classes D and E [n = 18]) (**A**) or the clinical outcome of infection, i.e., GBS (n = 14) versus uncomplicated gastroenteritis (ENT) (n = 34) (**B**). Experiments were performed in triplicate and repeated at least three times. For each strain, a geometric mean outcome (number of CFU per millilitre) was calculated. The differences between the geometric means of groups of strains were tested with the Mann-Whitney U statistic. The median for each group of strains is shown. Significant differences in invasion were observed between LOS classes ABC and DE * $p < 0.0001$ and GBS versus ENT * $p < 0.0046$

The invasiveness of the *C. jejuni* Penner serotype strains corresponded with LOS class expression of sialylated or non-sialylated LOS, with the exception of Penner serotype strain O:4. Thus, Penner serotype strain O:4 and also an enteritis-associated strain, Rivm 15, invaded poorly, despite the presumed expression of sialylated LOS due to the presence of a class A or C LOS biosynthesis gene cluster, respectively. Strain 81176 invaded the Caco-2 cell line as well as it did in previous studies, although most of those invasion studies were performed using a different cell line and a shorter incubation period (**Supplemental Table 1**). All Dutch clinical strains that contain LOS genes of class A, B, or C are thought to express sialylated LOS (12). Characterization of the LOS ganglioside mimic structures and determination of the presence or absence of sialylation for the GBS strains (GB2, GB3, GB4, GB11, GB13, GB17, GB19, GB22, GB23, GB25, and GB31) and enteritis strains (E98-623, 624, 652, 682, 706, 1033, and 1087) were carried out previously by immunological methods (1, 13), (**Supplemental Table 1**).

LOS phenotype characteristics of different *C. jejuni* strains and Δ*cst-II* mutants. As determined by mass spectrometry analysis, GB19 expressed sialylated LOS in the form of ganglioside mimic GD1c (also referred to as GD3, due to the structural similarity to human GD3). GD1c contains disialic acid bound to the terminal galactose residue. All three Δ*cst*-II mutants were chemically defined and found not to express sialylated LOS. The LOS structures of *C. jejuni* strains GB2, GB11, and GB19 and their associated Δ*cst*-II mutants are shown in **Fig. 2**.

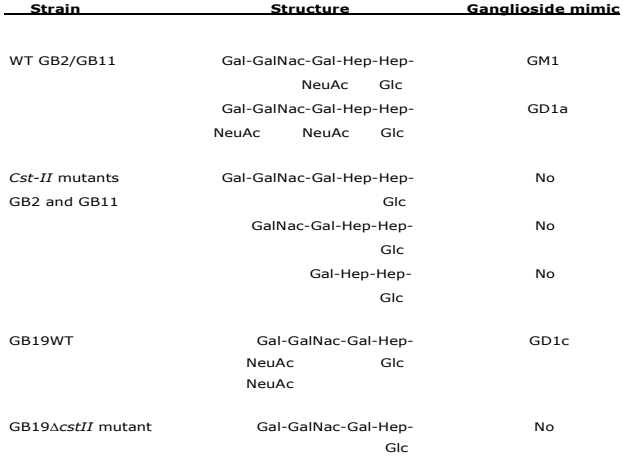

Figure 2 Proposed LOS outer core structures as determined by mass spectrometry analysis. Note that GB2 and GB11 express a mixture of the sialylated LOS ganglioside mimics GM1 and GD1a, whereas GB19 expresses sialylated LOS only in the form of GD1c. In all three strains, knockout mutagenesis of cst-II resulted in loss of expression of sialylated LOS.

For a subset of strains, comprising GB3, GB4, GB13, GB17, GB22, GB23, GB25, and GB31, ganglioside mimic structures were determined previously by mass spectrometry (**Supplemental Table 1**) (13). The LOS structures of the Penner serotype strains O:1, O:2, O:3, O:4, O:10, O:19, and 81176 (**Supplemental Table 1**) have been characterized previously by other researchers [15, 29-33]. As can be seen by the absence of data for some strains in (**Supplemental Table 1**), mass spectrometry data on LOS structures were not available for all bacteria.

Knock-out mutagenesis of *cst-II* does not significantly affect bacterial growth rate. To exclude the possibility that differences in viability and growth rates would influence the results of our invasion assays, we assessed the growth rates of wild-type strains GB2, GB11, and GB19 and their Δ*cst*-II mutants in Mueller-Hinton medium and in the cell culture medium used in the Caco-2 cell invasion assays. No significant differences in growth rates were observed between the wild-type GB2, GB11, and GB19 strains and their Δ*cst*-II mutants during the time span of our invasion experiments (data not shown).

Disruption of *cst-II* significantly affects the invasiveness of *C. jejuni* into intestinal epithelial cells. We compared the capacities of the *C. jejuni* wild-type strains GB2, GB11, and

GB19 to adhere to and invade Caco-2 cells with those of their respective Δ*cst*-II mutants. At an MOI of 100, wild-type and mutant strains adhered equally well to the human Caco-2 cell line (**Fig. 3A**). The only exception was the GB11Δ*cst*-II strain, which displayed a lower level of adherence than wild-type GB11 ($p = 0.031$). GB2Δ*cst*-II, GB11Δ*cst*-II, and GB19Δ*cst*-II all showed significant reductions in invasiveness relative to that of their wild-type parent strain ($p = 0.005$, $p = 0.002$, and $p = 0.008$, respectively) (**Fig. 3B**).

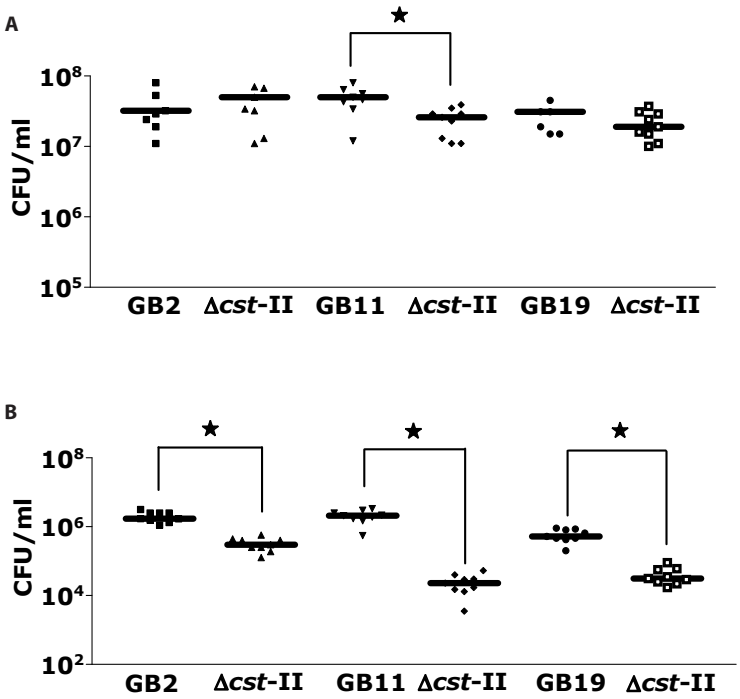

Figure 3. LOS sialylation plays an important role in invasion. *C. jejuni* wild-type strains GB2, GB11, and GB19 and their respective cst-II mutants were studied for adherence to (**A**) and invasion of (**B**) human enterocyte-like Caco-2 cells. Differences in adhesion and invasion were tested for significance by using the standard t test. Data are expressed as geometric means for 3 experiments each performed in triplicate. An astriks (*) indicates that a significant differences was detected.

In order to study whether the role of sialic acid in *C. jejuni* invasion is restricted to interactions with Caco-2 cells, a small selection of *C. jejuni* strains (P3, GB2, GB11, and GB13) and Δ*cst*-II mutants (GB2Δ*cst*-II and GB11Δ*cst*-II) were tested for invasiveness for the T84 human intestinal epithelial cell line (data not shown). The levels of invasiveness of all wild-type strains were similar in both cell types. Again, Δ*cst*-II mutants displayed reduced (by 1 to 1.5 log units) invasion of T84 cells. Together, these data establish that LOS sialylation contributes significantly to the invasion of intestinal epithelial cells by *C. jejuni*. We excluded variation in

microbial motility as the mechanism underlying the reduced invasion of the Δ*cst*-II mutant strains by performing quantitative swarming assays (data not shown).

Complementation of the GB11Δ*cst-II* mutant restores expression of sialylated LOS. Site-specific homologous recombination was used to reinstall the *cst-II* gene, together with its promoter region, in the GB11Δ*cst*-II strain. Using HRP-labelled cholera toxin as a detection agent, we confirmed the expression of sialylated LOS of the wild-type GB11 strain and of three selected clones of the complemented GB11Δ*cst*-II mutant by a Western blot assay (**Fig. 4**, lanes 1, 3, 4, and 5, respectively). The GB11Δ*cst*-II mutant did not express sialylated LOS (**Fig. 4**, lane 2). LOS isolated from the 11168 genome strain was used as a positive control for the binding of the HRP-labelled cholera toxin (**Fig. 4**, lane 6).

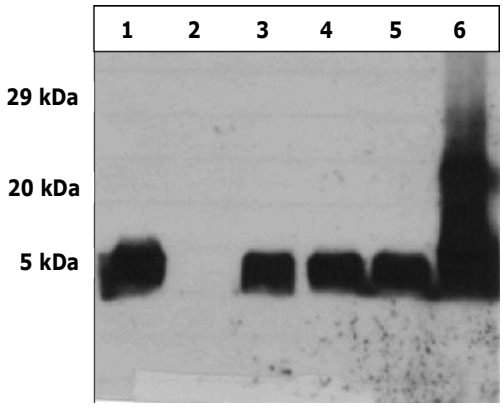

Figure 4 Cholera toxin confirms successful complementation. Western blot assay for analysis of cholera toxin binding; at the LOS of wild-type GB11, its Δ*cst*-II mutant, and the complemented GB11Δ*cst*-II mutant strain. Lane 1, LOS of the GB11 wild-type strain; lane 2,LOS of the GB11Δ*cst*-II mutant strain; lanes 3, 4, and 5, LOS from three selected clones of the complemented GB11Δ*cst-II* mutant; lane 6, LOS of the 11168 genome strain, used as a positive control. The LOS band is present at around 5 kDa.

Complementation of the GB11Δ*cst-II* mutant restores invasiveness. The Western blot assay provided evidence that the complemented mutant was now capable of LOS sialylation. With the gentamicin exclusion assay, we were able to show that this complementation also restored invasiveness to wild-type levels (**Fig. 5**). These results reiterate the importance of LOS sialylation in invasion.

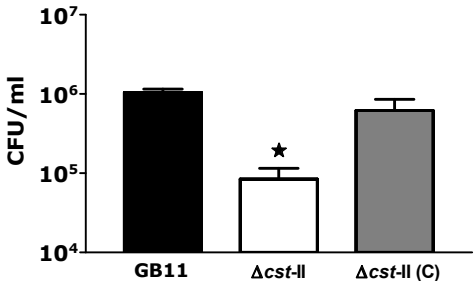

Figure 5 Complementation restores invasion phenotype. Complementation of the GB11 Δ*cst*-II mutant restores the wild-type phenotype for invasion observed with GB11. The *C. jejuni* wild-type strain GB11, the GB11Δ*cst*-II mutant, and the complemented GB11Δ*cst*-II (C) mutant were studied for invasion of human enterocyte-like Caco-2 cells. Data are geometric means from at least three independent experiments, each performed in duplicate. Error bars, standard deviations

Fixated sialylated LOS-containing strains inhibit invasion of their viable counterparts. The decreased invasiveness of GB2Δ*cst*-II, GB11Δ*cst*-II, and GB19Δ*cst*-II and the restored wild-type invasion phenotype of the complemented GB11Δ*cst*-II mutant clearly indicate a role for *C. jejuni* LOS sialylation in invasion. In order to further address the involvement of LOS sialylation in invasion, we designed an inhibition assay. We pre-incubated the Caco-2 cells with formalin-fixated, non-viable sialylated wild-type strains (GB2, GB11, and GB19) before incubating the cells with viable sialylated wild-type strains (GB2, GB11, and GB19). We found reductions of as much as 1 to 2 log units in invasion by viable wild-type strains. When Caco-2 cells were pre-incubated with an excess of formalin-fixated non-sialylated LOS Δ*cst*-II mutants, no differences in invasion were found relative to the invasion control (**Fig. 6**). The control groups consisted of Caco-2 cells that were incubated only with the viable wild-type strain GB2, GB11, or GB19. These results corroborate that LOS sialylation is an important determinant of epithelial cell invasiveness.

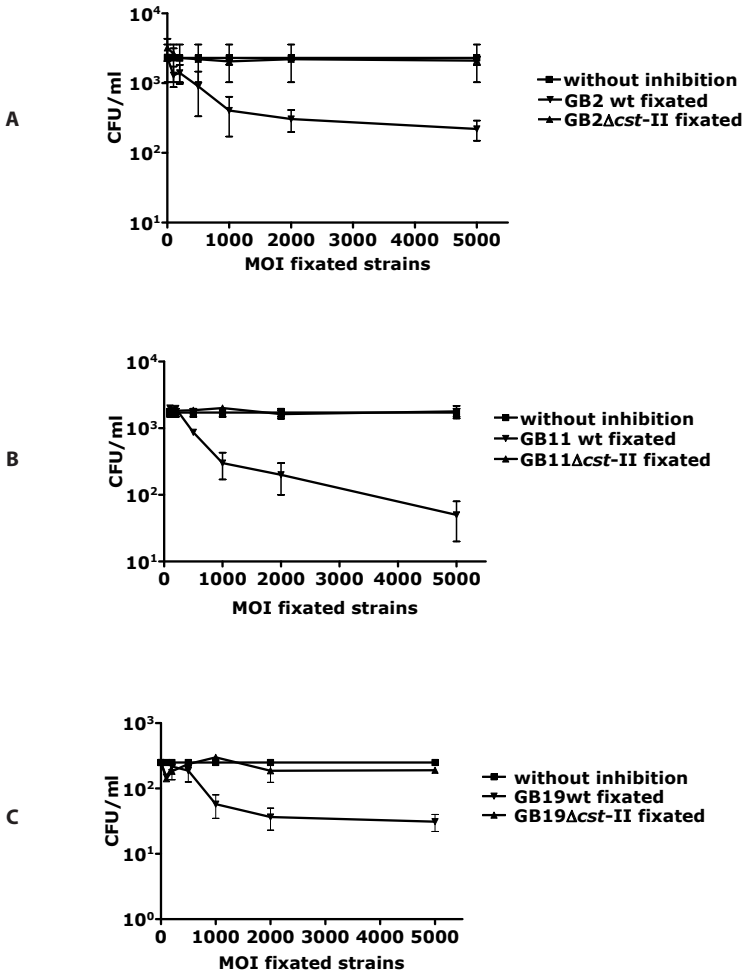

Figure 6 Blocking with fixated wild type and Δ*cst*-II mutant isolates confirms involvement of sialylated LOS in invasion. *C. jejuni* strains GB2, GB11, and GB19 invade Caco-2 cells via a sialylated-LOS-dependent mechanism(s). The levels of invasion by viable wild-type strains GB2 (**A**), GB11 (**B**), and GB19 (**C**) were assessed in the presence of either formalin-fixated GB2, GB11, or GB19 wild-type (wt) bacteria (sialylated LOS) or the respective fixated Δ*cst*-II mutants (truncated LOS, non-sialylated). Data are means from at least three independent experiments; error bars, standard deviations.

DISCUSSION

The mucosal epithelial cells are the first to interact with enteric pathogens such as *C. jejuni*. This microorganism may temporarily colonize the intestines in the absence of any clinical symptom. On the other hand, *C. jejuni* has been implicated in the pathogenesis of immune-mediated pathologies, e.g., GBS. Because *C. jejuni* infection can present with such a wide

range of symptoms, it is crucial to further identify factors and mechanisms that control *C. jejuni* epithelial invasion and persistence [34]. We hypothesized that the factors that regulate *C. jejuni* epithelial invasion may contribute directly to post-infectious sequelae, e.g., GBS.

Several *C. jejuni* outer membrane proteins, e.g., CadF, JlpA, and PEB1, play roles in epithelial adhesion and invasion [35-37]. Recently, PEB1 has also been identified as an amino acid transport system, which is essential for microbial growth [38]. Previous studies that identified microbial LOS as a generally important factor for invasion have been confirmed for *C. jejuni* [14-16, 18]. Here we specifically addressed if and to what extent sialylation of *C. jejuni* LOS contributes to microbial invasion. Therefore, we performed a large-scale survey by testing a heterogeneous panel of 48 human-isolated *C. jejuni* strains, 7 human control strains, and 3 sialyltransferase (*cst*-II) knockout strains. The knockout strains were previously shown to lack the capacity of LOS sialylation [25]. Our studies indicate that LOS sialylation facilitates epithelial invasion (**Supplemental Table 1**), since *C. jejuni* strains expressing sialylated LOS invaded significantly more frequently than non-sialylated LOS strains ($p < 0.0001$). Two strains with presumed LOS sialylation displayed low invasiveness. These results show that LOS sialylation must be regarded as an important contributor to *C. jejuni* invasiveness but not the single determinant. Earlier reports support the hypothesis that several factors determine invasiveness [14-16, 18]. Similar contributions of sialic acid to invasiveness have been established for other pathogens [39, 40]. In contrast, one study reports on inhibition of invasion by sialic acid [41].

Our experiments with the GB2, GB11, and GB19 sialyltransferase (*cst*-II) knockout strains further established the importance of LOS sialylation, since these mutated strains expressing non-sialylated LOS displayed significantly lower invasiveness than their respective wild-type controls. The methods for generation of such knockout strains may be accompanied by various technical side effects, e.g., mutation of genes other than the target gene. Furthermore, insertion of an antibiotic resistance cassette may induce expression or silencing of adjacent genes and gene products. Therefore, we set up experiments using a complemented Δ*cst*-II mutant strain. We show that this procedure indeed restored sialylation of the LOS (**Fig. 4**) and subsequent invasiveness to wild-type levels (**Fig. 5**).

In our studies, only the GB11Δ*cst*-II mutant strain showed diminished adherence relative to that of its wild-type parent strain, indicating a less important role for LOS sialylation in epithelial adhesion than in invasion. These findings indicate that adhesion and invasion are regulated by different sets of factors. Adhesion is likely established by proteins such as CadF, JlpA, and PEB1 [35-37], whereas invasion is more influenced by LOS sialylation in the strains we tested. To support the hypothesis that invasion is facilitated by LOS sialylation, we established that formalin-fixated wild-type strains GB2, GB11, and GB19, but not the isogenic *cst*-II mutants, were able to inhibit epithelial invasion by viable GB2, GB11, and GB19 strains. These findings may have two implications. First, these data may help to identify novel epithelial invasion receptors. Second, these experiments may lead to the discovery of specific agents that can be used to block microbial invasion.

Previously, sialylation of *C. jejuni* LOS was associated with GBS [25, 42-43]. Isolates from GBS patients mainly synthesize sialylated LOS of classes A and B (± 80%) [44]. Strains isolated from enteritis patients show a more mixed LOS composition, with a tendency toward non-sialylated LOS expressed by classes D and E. Notably, the presence of strains expressing LOS classes A and B in enteritis patients is around 20 to 25%. Therefore, the enhanced invasiveness of GBS-associated strains seems to result from the frequent presence of LOS class A and B strains in this patient group [45]. We hypothesize that among other risk factors, enhanced invasiveness (e.g., through LOS class A expression) contributes to the development of post-infectious complications such as GBS.

In conclusion, we demonstrate that *C. jejuni* strains expressing sialylated LOS have an overall increased capacity to invade intestinal epithelial cells. Knockout mutagenesis of the *cst*-II gene and complementation and blocking experiments provide additional evidence on the role of LOS sialylation in the invasion of the intestinal epithelium. Understanding the function of LOS sialylation in epithelial cell invasion may provide us with potential target structures for future therapeutic interventions in *C. jejuni*-mediated diarrhoeal disease and its post-infectious complications.

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EXPERIMENTAL METHODS

Bacterial strains. 14 GBS- and 34 enteritis-associated *C. jejuni* strains, isolated from Dutch patients, 6 Penner serotype strains and the 81176 enteritis reference strain, were used in this study (Supplementary Results, Data File 1). To minimize in vitro passages, *C. jejuni* strains were recovered from the original patient isolated glycerol stock by culturing on Butzler agar plates (Becton Dickinson, Breda, The Netherlands). A second passage was allowed for optimal vitality before using these strains in experiments. After recovery cells were harvested in Hanks

Balanced Salt Solution (HBSS) (Life Technology, Breda, The Netherlands) and densities were adjusted according to the optical density (OD) at 600 nm.

Typing of the LOS biosynthesis gene cluster. To determine the class of LOS locus present in each *C. jejuni* strain, genomic DNA was isolated using the DNeasy Tissue kit (Qiagen, Venlo, The Netherlands). PCR analysis was done with primer sets specific for the classes A, B, C, D and E as previously described [25]. PCR assays were performed in a Perkin Elmer GeneAmp PCR System 9700 (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands), applying 35 cycles of 1 min 94°C, 1 min 52°C, 2 min 72°C.

Knock-out mutagenesis. Strains GB2, GB11 and their Δ*cst-II* mutants, GB2Δ*cst-II* and GB11Δ*cst-II*, respectively, have been described before [25]. A Δ*cst-II* mutant of a third GBS-related strain that is described here, GB19, was generated using the same procedure used for the knock-out mutagenesis in strains GB2 and GB11 [25]. Briefly, the target gene (*cst-II*) and approximately 700 bp of upstream and downstream flanking sequences were amplified and cloned into the pGem-Teasy vector (Promega Corp, Leiden, The Netherlands). Inverse PCR was used to introduce a *BamHI* restriction site and a deletion of approximately 800bp in the target gene. Inverse PCR products were digested with *BamHI* (Fermentas, St. Leon-Rot, Germany) and ligated to the *BamHI* digested chloramphenicol resistance (Cm^r) cassette. Constructs were electroporated into electrocompetent GB19 *C. jejuni* cells and recombinants were selected on Mueller-Hinton plates (Becton Dickinson, Breda, The Netherlands) containing 20μg/ml chloramphenicol (Difco, Alphen aan den Rijn, The Netherlands).

Mass spectrometry. Samples were prepared for LOS mass-spectrometric analysis by overnight growth of *C. jejuni* strains at 37°C on Butzler agar plates in a micro-aerobic atmosphere. Material from one confluent agar plate in a micro-aerobic atmosphere was harvested and treated with proteinase K at 60 μg/ml, RNase A at 200 μg/ml, and DNase I at 100 μg/ml (Promega, Leiden, The Netherlands). *O*-deacylated LOS samples were prepared and analyzed by capillary electrophoresis coupled to electro-spray ionization mass spectrometry (CE-ESI-MS) [46].

Complementation of the *cst-II* gene. We used site specific homologous recombination to restore the wild type phenotype of the GB11Δ*cst-II* mutant strain (manuscript in preparation). In short, a construct containing the *cst-II* gene together with its promoter region and a gene encoding erythromycin resistance were cloned in the same orientation and were transformed by electroporation into electrocompetent GB11Δ*cst-II* mutant cells. The electroporated cells were plated on selective blood agar plates containing 10μg/ml erythromycin (Sigma Aldrich, Zwijndrecht, The Netherlands) and incubated at 42°C in a micro-aerobic environment. Colonies formed were sub-cultured to purity and stored at -80°C until further use.

SDS-PAGE and Western blot assay. To analyze *C. jejuni* LOS sialylation, a 10% SDS-PAGE gel was run. Strains were harvested from an overnight Butzler agar plate, where after concentrations were equalized by OD 600 nm measurement. Bacterial cell suspensions were lysed using glass beads (MP Biomedicals, Solon, OH, USA). Lysates were digested with proteinase K

at 60 µg/ml for 4 hours at 56 ^{0}C and equal amounts were run on a 10% SDS-PAGE Tris-HCl gel for 2 hours. As a standard the pre-stained SDS-PAGE broad range molecular weighted marker was used (Bio-Rad, Nazareth Eke, Belgium). After electrophoresis, the LOS was transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) for a Western-blot assay. The nitrocellulose membrane was blocked overnight with 0.05% (v/v) Tween-20 (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 5% (W/V) nonfat milk (Bio-Rad, Nazareth Eke, Belgium). The next day the membranes were washed three times for 10 minutes with PBS and incubated with horse radish peroxidase (HRP) labeled cholera toxin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 1% blocking buffer as a detection agent. Presence or absence of sialylated LOS was visualized with an ECL detection kit (Biocompare, San Francisco, USA) and a Kodak photo film (Roche-Diagnostics, Almere, The Netherlands) according to the manufacturer's protocol.

Bacterial growth assay. Bacterial growth characteristics of the clinical isolates and their corresponding mutants were determined in Mueller-Hinton broth (Becton Dickinson, Breda, The Netherlands) and in a specific antibiotic-free cell culture medium, which is used in the gentamicin exclusion assay. Bacterial strains were inoculated at equal OD at 600 nm, equivalent to 5.0 x 10^4 CFU/ml, and incubated at 37 ^{0}C, while gently shaking in a micro-aerobic environment. Bacterial cell counts and OD 600 nm were determined at 4, 8, 18, 24, 36 and 42 hours post-inoculation, respectively.

Intestinal epithelial cell line. Human intestinal epithelial Caco-2 and T84 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Breda, The Netherlands) and 1% non-essential amino acids (NEAA) (Invitrogen, Breda, The Netherlands). The cells were routinely grown in a 75-cm^2 flask (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) at 37 ^{0}C in a 5% CO_2 and 95% air humidified incubator. Confluent stock cultures were washed with phosphate buffered saline (PBS) (Invitrogen, Breda, The Netherlands), trypsinized with Tripsene-Versene (Lonza, Verviers, Belgium) and 5.0 x10^5 cells were seeded into a new 75-cm^2 flask.

Adhesion and invasion. Adherence and invasion of *C. jejuni* was determined by growing the intestinal epithelial cells (Caco-2 or T84) to confluence for 48 hours at a final approximate density of 5.0 x10^6 cells per well (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), without allowing them to differentiate in the case of Caco-2 cells. The adherence and invasion assays were performed by incubating the epithelial cells with *C. jejuni* at a ratio of 1:100. Bacteria and epithelial cells were co-incubated for 2 hours at 37 ^{0}C in a 5% CO_2 and 95% air atmosphere to assess adherence. For invasion, a subsequent 2 hours of incubation of the epithelial cells was allowed. After incubation, monolayers were washed 3 times with pre-warmed PBS. To kill extra-cellular bacteria, monolayers were treated for 3 hours with a bactericidal concentration of gentamicin (480 µg/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) in DMEM medium containing 10% FBS and 1% NEAA as described previously [8]. For all strains, sensitivity to this

concentration of gentamicin was confirmed. After washing, epithelial cells were lysed with 0.1% Triton X-100 (Cornell, Philadelphia, PA, USA) in PBS for 15 minutes at room temperature. The number of invaded *C. jejuni* was determined by plating serial dilutions of the lysis mix onto freshly prepared blood agar plates. After incubation for 24-36 hours at 37 ^{0}C in a micro-aerobic environment, colonies were counted. The percentage of bacteria that invaded was calculated by dividing the number of *C. jejuni* that invaded the cells by the number of *C. jejuni* inoculated onto the cells times 100%. For determination of adherence, cells were washed three times extensively with PBS and the cell monolayer was lysed with 0.1% Triton X-100 after which serial dilutions were plated onto blood agar plates (Becton Dickinson, Breda, The Netherlands).

Inhibition of invasion. Formalin fixated, wild type *C. jejuni* and their Δ*cst-II* mutants were used to inhibit invasion of viable *C. jejuni* GB2, GB11 and GB19. Briefly, GB2, GB11, GB19 and their Δ*cst-II* mutants at a starting concentration of 5.0 x10^9 CFU/ml, determined at OD 600 nm, were fixated in 3.6% formalin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS for 10 minutes. By washing the fixated cells 3 times in PBS the excess of formalin was removed. The sterility of the control cultures confirmed fixation to be complete. Caco-2 cells at a density of 5.0 x10^4 cells per well were pre-incubated for 30 minutes with formalin-killed wild type or Δ*cst-II* mutant *C. jejuni* at a multiplicity of infection (MOI) ranging from 100 to 5000. Subsequently, the Caco-2 cells were washed to remove excess dead *C. jejuni* bacteria where after fresh medium was added. Viable wild type cells were added at a MOI of 100 and invasion was assessed by the gentamicin exclusion protocol as described earlier.

Statistical analysis. Statistical analysis was performed using Instat™ software (Graphpad Software version 2.05a, San Diego, CA). As invasiveness of strains varied widely, log-transformation was used to equalize variances. Invasiveness was expressed as the geometric mean number of CFUs/ml retrieved from the infected cell-line in all three to six invasion experiments per *C. jejuni* strain performed. Differences in invasiveness between LOS class A, B and C versus LOS class D and E strains and GBS- versus enteritis-associated strains were tested for significance with a Mann Whitney U test as column statistics showed that the Gaussian distribution was unequal for the strains. A two-tailed value smaller than $p < 0.05$ indicated statistical significance. Statistical analysis for difference in adherence and invasion between wild type and knock-out mutant was tested for significance with a paired t-test.

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Supplemental Table 1

STRAINS [1]	LOS locus [2]	Invasion % [3]	*C. jejuni* per 100 cells [4]	Ganglioside mimic [5]	Illness [6]
GB2	A	3.4 ± 0.55	285 - 395	GM1a, GD1a	GBS
GB11	A	2.2 ± 0.7	150 - 290	GM1a, GD1a	GBS
GB19	A	0.8 ± 0.29	51 - 109	GD1c	GBS
GB3	A	0.12 ± 0.046	7 - 16	GM1a, GD1a	GBS
GB22	A	0.05 ± 0.026	3 - 7	GM1a, GD1a	GBS
GB23	A	1.17 ± 0.14	103 - 131	GM2	GBS
GB29	A	0.73 ± 0.06	67 – 79		GBS
E990521	A	3.0 ± 1.15	185 - 415		Enteritis
E991095	A	1.9 ± 0.81	110 - 271		Enteritis
E9126	A	1.2 ± 0.58	70 - 178		Enteritis
P19	A	4.7 ± 1.4	330 - 610	GM1a, GD1a	Enteritis
P10	A	4.23 ± 1.86	237 - 609	GD3	Enteritis
P4	A	0.0054 ± 0.00092	0.44 – 0.63	GM1a, GD1a	Enteritis
GB17	B	3.05 ± 1.75	130 – 480	GM1b, GD1c	GBS
GB25	B	0.27 ± 0.13	14 - 40	GM1b, GD1c	GBS
GB31	B	0.97 ± 0.15	82 – 112	GM1a, GD1a	GBS
GB37	B	0.16 ± 0.03	13 – 19		GBS
Rivm 16	B	1.98 ± 0.7	192 – 205		Enteritis
Rivm 38	B	0.037 ± 0.023	1.0 – 6.0		Enteritis
Rivm 129	B	0.084 ± 0.026	5.0 – 11		Enteritis
E989123	B	0.29 ± 0.011	18 – 40		Enteritis
E981033	B	0.26± 0.075	18 - 33	GM1a	Enteritis
E98652	B	0.028 ± 0.006	2 - 4	GM1a, GQ1b	Enteritis
81176	B	0.26 ± 0.06	20 – 32	GM2, GM3	Enteritis
GB13	C	0.2 ± 0.017	18 - 22	GM1a	GBS
GB38	C	1.8 ± 0.77	103 – 257		GBS
Rivm 15	C	0.00075 ± 0.00014	0.061 – 0.089		Enteritis
Rivm 83	C	2.75 ± 1.28	147 – 403		Enteritis
Rivm 93	C	3.5 ± 1.15	235 – 465		Enteritis
Rivm 109	C	1.22 ± 0.44	78 – 166		Enteritis
Rivm 116	C	0.25 ± 0.13	12 – 38	GM1a. GQ1b	Enteritis
E98682	C	0.010 ± 0.0036	0.6 – 1.4	GM1a	Enteritis
E981087	C	0.13 ± 0.031	10 - 16	GM2	Enteritis
P1	C	0.01 ± 0.001	0.9 – 1.1	GM1b	Enteritis
P2	C	0.005 ± 0.0017	0.33 – 0.67		Enteritis
Rivm 3	D	0.005± 0.0012	0.38 – 0.62		Enteritis
Rivm 33	D	0.017 ± 0.0045	1 – 2		Enteritis
Rivm 65	D	0.018 ± 0.0026	1 – 2		Enteritis
Rivm 67	D	0.0097 ± 0.0013	0.5 – 1		Enteritis
Rivm 95	D	0.019 ± 0.003	1 – 2		Enteritis
Rivm 104	D	0.0082 ± 0.0014	0.68 – 0.96	none	Enteritis
E98706	D	0.014 ± 0.0025	1.15 – 1.65		Enteritis
E970873	D	0.14 ± 0.02	12 – 16	none	Enteritis
GB4	E	0.009 ± 0.003	0.5 – 1		GBS

Supplemental Table 1 (continued)

STRAINS [1]	LOS locus [2]	Invasion % [3]	*C. jejuni* per 100 cells [4]	Ganglioside mimic [5]	Illness [6]
Rivm 37	E	0.081 ± 0.029	5 – 11		Enteritis
Rivm 46	E	0.0065 ± 0.0027	0.38 – 0.92		Enteritis
Rivm 47	E	0.097 ± 0.028	6 – 12		Enteritis
Rivm 50	E	0.0065 ± 0.00096	0.56 – 0.74		Enteritis
Rivm 61	E	0.011 ± 0.0066	1 – 2		Enteritis
E9141	E	0.074 ± 0.013	5 - 9		Enteritis
E9144	E	0.14 ± 0.03	11 - 17		Enteritis
E9146	E	0.08 ± 0.015	6 - 10	none	Enteritis
E98623	E	0.004 ± 0.0015	0.2 – 0.5	none	Enteritis
E98624	E	0.003 ± 0.00075	0.23 – 0.4	none	Enteritis
P3	E	0.0045 ± 0.0013	0.32 – 0.58		Enteritis

Supplemental Table 1 *C. jejuni* strains and their invasiveness into Caco-2 cells. [1] Strains used in the invasion assay; GB are the GBS-associated isolates; RIVM and E are the enteritis-associated isolates; P are the Penner typed isolates used as a control in this study; [2] LOS locus shows the LOS class detected by PCR as described earlier [25]; [3] Invasion % shows the amount of *C. jejuni* bacteria recovered from the Caco-2 cells displayed in percentage; [4] Shows the average amount on number of *C. jejuni* bacteria per Caco-2 cell; [5] ganglioside mimics detected by mass spectrometry, not established yet for all the isolates; [6] Outcome of disease induced by *C. jejuni* enteritis only or accompanied by the post-infectious complication GBS.

Chapter 4

Campylobacter jejuni translocation across intestinal epithelial cells is facilitated by ganglioside-like lipooligosaccharide structures.

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ABSTRACT

Translocation across intestinal epithelial cells is an established pathogenic feature of the zoonotic bacterium *Campylobacter jejuni*. The *C. jejuni* virulence factors known to be involved in translocation are limited to only a few. In the present study we investigated whether sialylation of *C. jejuni* lipo-oligosaccharide (LOS) structures, structures that mimic human nerve gangliosides, are important for intestinal epithelial translocation. We first of all show that *C. jejuni* isolates expressing ganglioside mimic structures bound in elevated numbers onto Caco-2 intestinal epithelial cells in comparison to *C. jejuni* isolates lacking ganglioside mimic structures. Next, we found that *C. jejuni* ganglioside mimic expression facilitated Caco-2 intestinal epithelial cell endocytosis visualized by quantitative microscopic analysis using the early and late endosomal markers EEA1, Rab5, Rab7 and LAMP-1. Increased endocytosis as observed for ganglioside mimic expressing *C. jejuni* isolates was associated with increased numbers of translocating bacteria. In response to this more severe infection, we found that two different intestinal epithelial cell lines (Caco-2 and T84) reacted both with an elevated epithelial release of the T-cell attractant CXCL10, when challenged with ganglioside mimic expressing *C. jejuni* isolates. We conclude that *C. jejuni* translocation across intestinal epithelial cells is facilitated by ganglioside-like LOS, which is of interest since *C. jejuni* ganglioside mimic expressing isolates are linked with severe gastro-enteritis and bloody stools in *C. jejuni* diseased patients.

INTRODUCTION

Campylobacter jejuni, a zoonotic Gram-negative human bacterial pathogen, is able to enter, survive and translocate across intestinal epithelial cells [1-3]. Bacterial pathogens such as *C. jejuni* that enter mammalian cells often employ common eukaryotic cellular pathways such as endocytosis [1, 3-6]. Endocytosis provides the general entry portal of eukaryotic cells for uptake of nutrients and regulation of membrane-bound receptors and signalling [7]. Endocytosis consists of early and later stages that can be conveniently distinguished using specific protein markers. The protein markers frequently used to study the different endocytic stages are the early-endosome associated protein 1 (EEA1), the GTPase proteins Rab5 and Rab7 and the lysosomal-associated membrane protein 1 (LAMP-1). EEA1 and Rab5 are involved in the early stages of endocytosis [8], Rab7 marks later endocytosis stages [9], whereas LAMP-1 marks the end stage, when late endosomes are fused with lysosomes [10,11]. At the final stages of endocytosis, endo-lysosomal vesicles, organelles in which large molecules and even intact bacteria can be degraded, are formed [12, 13].

Earlier, the extensively studied *C. jejuni* isolate 81176 was found to translocate across intestinal epithelial cells via transcytosis (apical endocytosis and basolateral exocytosis) [1] and almost at the same time shown by others to escape lysosomal killing [3]. Overall, the *C. jejuni* factors known to be involved in transcytosis, lysosomal escape and translocation are limited to only a few [14, 15]. Of interest to us was therefore the study showing that sialylation of *C. jejuni* lipo-oligosaccharide (LOS) structures, structures that mimic human peripheral nerve gangliosides [16], associated with severe gastro-enteritis and bloody stools in *C. jejuni* diseased patients [17]. Since the ganglioside mimic expressing isolate 81176 [18] is also able to induce severe *coli*tis in humans [19], we searched *in vitro* for a potential pathogenic mechanism to be able to explain this association. We hypothesized that severe gastro-enteritis could be a result of increased numbers of endocytosed and translocating bacteria in the human intestine. The present work was therefore designed to explore the effect of ganglioside-like LOS on *C. jejuni* endocytosis, intra-cellular survival and translocation across human intestinal epithelial cells.

RESULTS

Ganglioside-like LOS increases the number of *C. jejuni* bacteria bound to Caco-2 intestinal epithelial cells. We determined if sialylation of *C. jejuni* LOS structures, in other words ganglioside mimic expression (GM+), had an effect on binding to Caco-2 intestinal epithelial cells. Microscopic analysis of Caco-2 intestinal epithelial cells during the first 10 - 15 min of infection revealed that the GM+ isolate 81176 bound more efficiently then the isolate R104, which lacks sialylated LOS (GM-) (**Fig. 1A and 1B**). Quantitative microscopic analysis showed a 3 fold enhanced binding capacity of the *C. jejuni* isolates GB11, GB19 and 81176 (GM+) to Caco-2 intestinal epithelial cells compared to the non-sialylated *C. jejuni* isolates R104, R65 and 9141 (GM-) ($p < 0.0001$) (**Fig. 1C**). To confirm the effect of *C. jejuni* ganglioside mimic expression on Caco-2 intestinal epithelial cell binding, we tested whether Δ*cst*-II mutants (GM-) were less able to bind as compared to their respective wild-type isolates. Microscopic analysis revealed that the wild type isolate GB11 bound more efficient to the Caco-2 intestinal epithelial cells as compared to its Δ*cst*-II mutant, respectively (**Fig. 1D and 1E**). Quantitative microscopic analysis revealed that the GB11Δ*cst*-II and GB19Δ*cst*-II mutants nearly lost all capacity to bind Caco-2 intestinal epithelial cells, in strong contrast with their corresponding wild-type isolates ($p < 0.0001$) (**Fig. 1F**). These results reveal that ganglioside mimic expression contributes strongly to *C. jejuni* binding of intestinal epithelial cells during the first 10 - 15 minutes of infection.

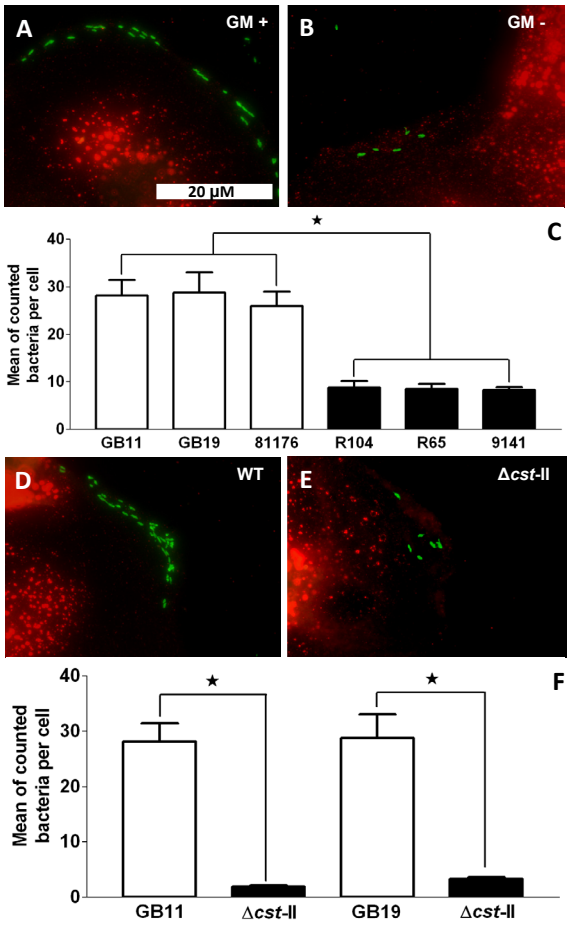

Figure 1 Ganglioside-like LOS increases the number of *C. jejuni* bacteria bound to Caco-2 intestinal epithelial cells. Caco-2 cells were incubated with *C. jejuni* for 10 - 15 minutes, paraformaldehyde fixated and processed for immunofluorescence using anti-*C. jejuni* FITC-labelled antibody (green). **A** and **D**) shows the binding of the GM+ isolates 81176 and GB11 to a Caco-2 cell, respectively. **B** and **E**) shows the binding of the GM- isolate R104 and the GB11Δ*cst*-II mutant (thus functionally GM-), respectively. **C)** shows the mean binding per cell for the GM+ isolates (white bars) and GM- isolates (black bars); error bars show the standard error of the mean of three independent experiments. The difference in binding between GM+ and GM- isolates were significant (Mann-Whitney U test, * $p < 0.0001$). **F**) shows the mean binding per cell for the wild type isolates (white bars, GM+) and their Δ*cst*-II mutants (black bars, GM-); error bars show the standard error of the mean of three independent experiments. The difference in cellular binding between wild type and Δ*cst*-II mutants were significant (Paired t-test, * $p < 0.0001$).

Ganglioside-like LOS enhances *C. jejuni* endocytosis. To further explore the involvement of ganglioside mimic expression during the early infection cycle, we determined co-localization of our isolates with the early endosomal markers EEA1 and Rab5. Quantitative microscopic analysis revealed that the GM+ isolate 81176 co-localized in enhanced numbers with the early endosomal markers EEA1 and Rab5 (**Fig. 2A - C** and **Fig. 3A - C**) compared to a

GM- isolate R104 (**Fig. 2D - F** and **Fig. 3D - F**). In the time frame used to study co-localization of *C. jejuni* isolates (GM+ and GM-) with EEA1 and Rab5, we again observed that *C. jejuni* ganglioside mimic expression contributed to cellular binding during infection of the Caco-2 cells (**Fig. 2G** and **3G** and **Fig. 2H** and **3H**). Determining the level of bacterial co-localization with the early endosomal markers EEA1 and Rab5 further showed that ganglioside mimic dependent cellular binding by *C. jejuni* facilitated cellular entry via the endosomal pathway. The GM+ isolates GB11, GB19 and 81176 showed enhanced co-localization with these early endosomal markers compared to the GM- isolates R104, R65 and 9141 ($p < 0.0001$; **Fig. 2I** and **Fig. 3I**). Comparing 2 Δ*cst*-II mutants (GM-) with their wild type isolates (GM+) unambiguously established that the differences in co-localization with EEA1 and Rab5 depended on *C. jejuni* LOS ganglioside mimic expression (**Fig. 2J** and **Fig. 3J**).

Rab7 is not involved in *C. jejuni* endocytosis. The Rab7 protein is a marker for later stages of endosomal trafficking [25], but is not essential for endosome maturation preceding fusion with lysosomes [26]. Based on an earlier study using the 81176 isolate (GM+) [3], we first tested if Rab7 co-localized with this *C. jejuni* isolate. We did not observe significant co-localization of the 81176 isolates with Rab7 in Caco-2 cells (**Fig. 4A - C**).

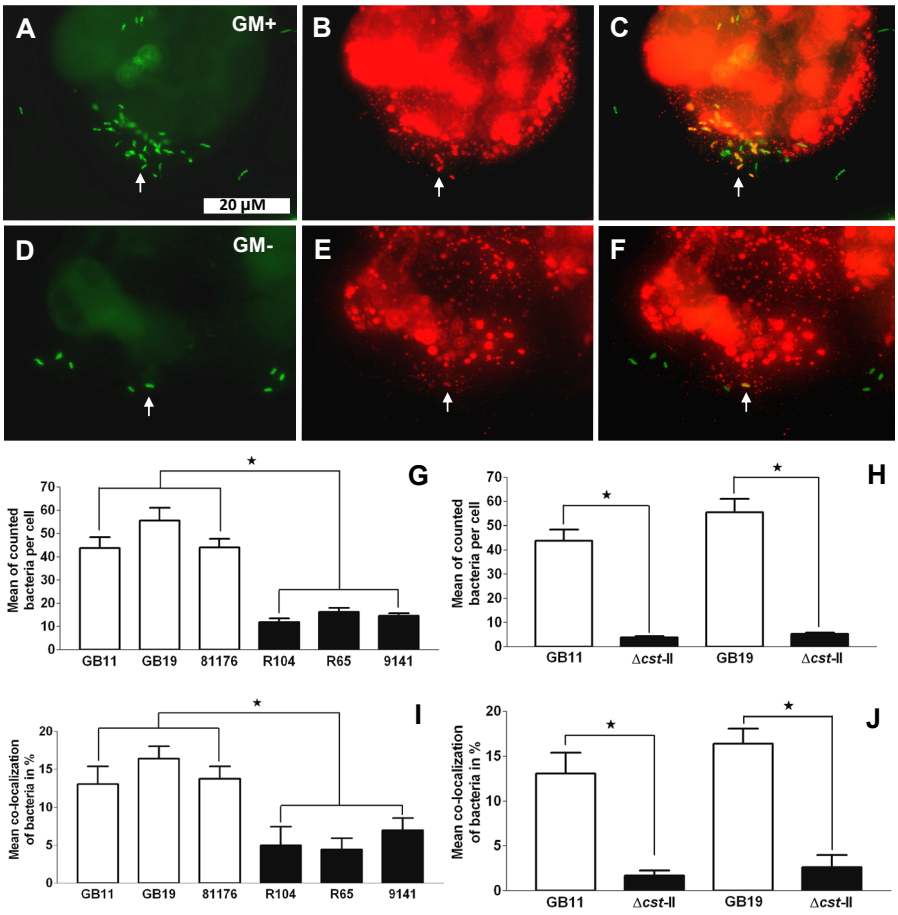

Figure 2 *C. jejuni* ganglioside-like LOS enhances the number of EEA1 co-localizing bacteria. Caco-2 cells were incubated with *C. jejuni*, paraformaldehyde fixated at 30 - 35 minutes and processed for immuno-fluorescence using, **A** and **D**) anti-*C. jejuni* FITC labelled (green) and **B** and **E**) anti-EEA1 antibodies (red), **C** and **F**) show merging of **A** and **B** and **D** and **E** to visualize the EEA1 co-localization efficiency of GM+ versus GM- isolates. White arrow shows example of co-localization. **G** and **H** show the mean number of *C. jejuni* bacteria per cell; the error bars show the standard error of the mean of three independent experiments**.** Significant differences in cellular binding were observed between GM+ isolates (white bars), GM- isolates (black bars) (Mann-Whitney U test, $^{*}p < 0.0001$) and between wild type (white bars, GM+) and Δ*cst*-II mutants (black bars, GM-) (Paired t-test, $^{*}p < 0.0001$). **I**) and **J)** EEA1 co-localization of *C. jejuni* was quantified for GM+ (white bars), GM- (black bars) and Δ*cst*-II mutants (black bars, GM-). Results are shown as the mean percentage of EEA1 co-localizing bacteria per Caco-2 cell; error bars show the standard error of the mean of three independent experiments. Significant difference were established in EEA1 co-localization between GM+ and GM- wild type isolates (Mann-Whitney U test, $^{*}p < 0.0001$) and between wild type and Δ*cst*-II mutants (Paired t-test, $^{*}p < 0.0001$).

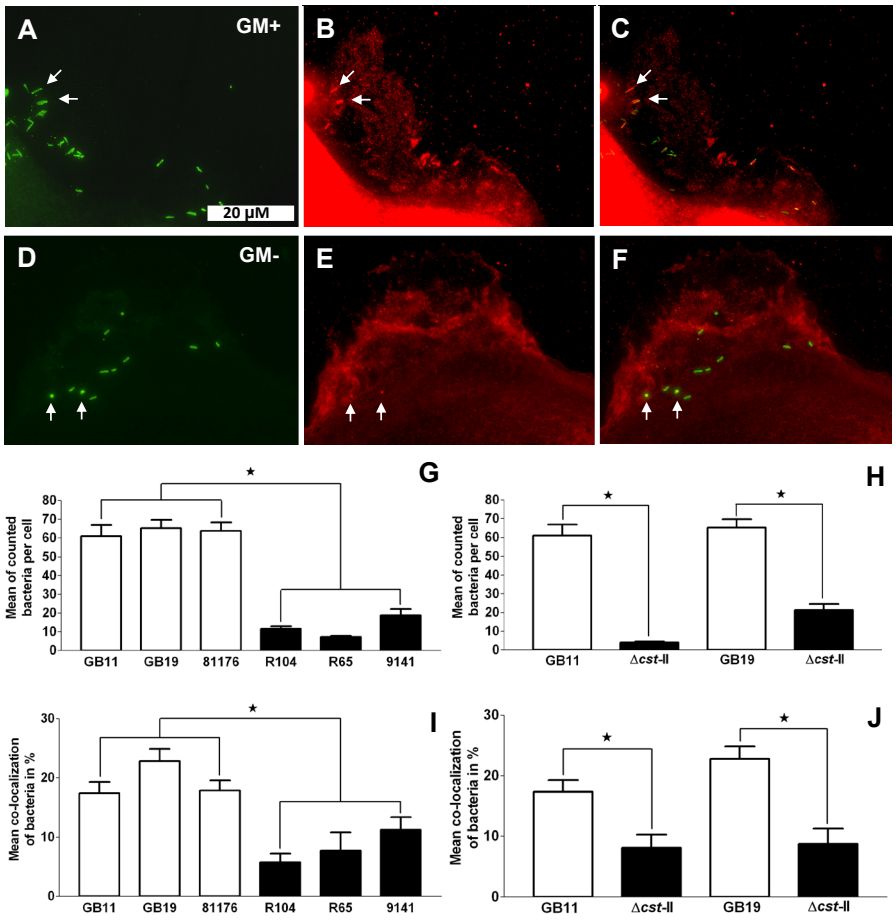

Figure 3 *C. jejuni* ganglioside-like LOS enhances the number of Rab5 co-localizing bacteria. Caco-2 cells were incubated with *C. jejuni*, paraformaldehyde fixated at 35 - 40 minutes and processed for immuno-fluorescence using, **A** and **D**) anti-*C. jejuni* FITC labelled (green) and **B** and **E**) anti-Rab5 antibodies (red), **C** and **F**) show merging of **A** and **B** and **D** and **E** to visualize the Rab5 co-localization efficiency of GM+ versus GM- isolates. White arrow shows example of co-localization. **G** and **H** show the mean number of *C. jejuni* bacteria per cell; the error bars show the standard error of the mean of three independent experiments**.** Significant differences in cellular binding were observed between GM+ isolates (white bars) and GM- isolates (black bars) (Mann-Whitney U test, $^{*}p < 0.0001$) and wild type (white bars) and Δ*cst*-II mutants (black bars, GM-) (Paired t-test, $^{*}p < 0.0001$). **I**) and **J)** Rab5 co-localization of *C. jejuni* was quantified for GM+ (white bars), GM- (black bars) and Δ*cst*-II mutants (black bars, GM-). Results are shown as the mean percentage of Rab5 co-localizing bacteria per Caco-2 cell; error bars show the standard error of the mean of three independent experiments. Significant difference were established in Rab5 co-localization between GM+ and GM- wild type isolates (Mann-Whitney U test, $^{*}p < 0.0001$) and wild type and Δ*cst*-II mutants (Paired t-test, $^{*}p < 0.0001$).

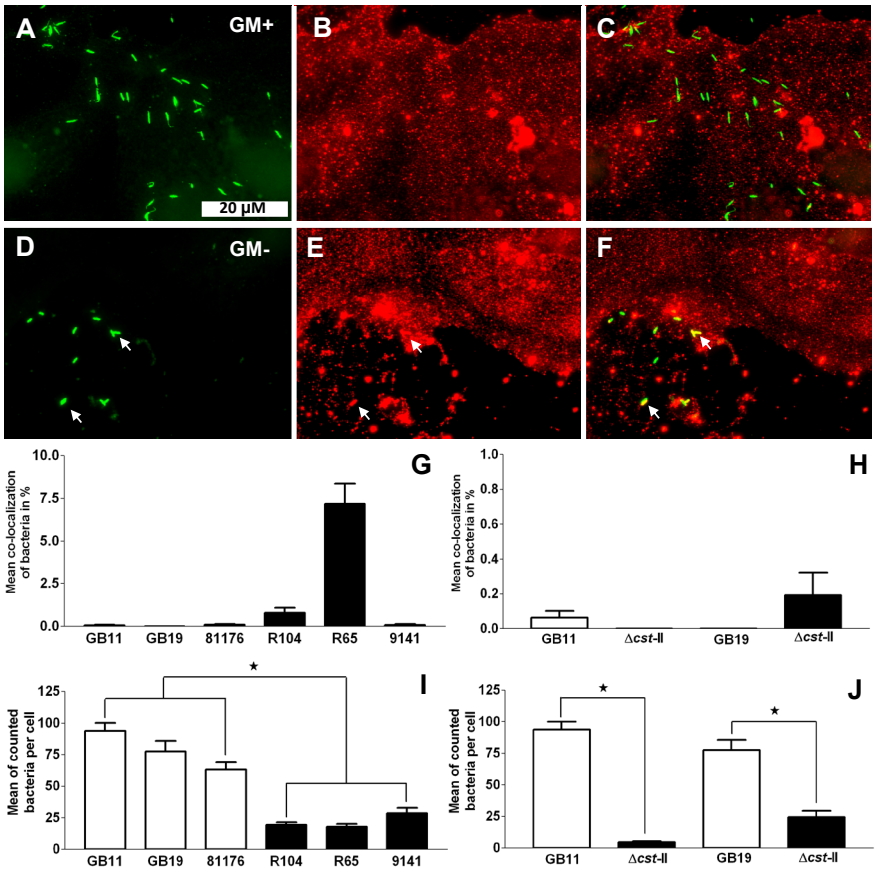

Figure 4 Rab7 is not involved in *C. jejuni* endocytosis. Caco-2 cells were incubated with *C. jejuni*, paraformaldehyde fixated at 40 - 45 minutes and processed for immuno-fluorescence using, **A** and **D**) anti-*C. jejuni* FITC labelled (green), **B** and **E**) anti-Rab7 antibodies (red), **C** and **F**) **A** and **B** and **D** and **E** were merged to visualize the Rab7 co-localization efficiency of GM+ versus GM- isolates, white arrow shows an example of co-localization **G**) and **H)** Results are shown as the mean percentage of Rab7 co-localizing bacteria per cell; error bars show the standard error of the mean of three independent experiments. The difference in co-localization with Rab7 between GM+ (white bars) versus GM- isolates (black bars) and wild type (GM+) and Δ*cst*-II mutants (GM-) were not significant. **I)** and **J)** show the mean number of *C. jejuni* bacteria per cell; the error bars show the standard error of the mean of three independent experiments**.** Significant differences in cellular binding were observed between GM+ isolates and GM- wild type isolates (Mann-Whitney U test, $^{*}p < 0.0001$) and between wild type and Δ*cst*-II mutants (Paired t-test, $^{*}p < 0.0001$).

Therefore, all GM+ and GM- isolates used in this study were tested at different time points between 15 minutes and 2 hours after inoculation for co-localization with Rab7. We only detected a significant increase in co-localization with Rab7 after 40 minutes for the isolate R65 (GM-) (**Fig. 4D - F**). For the other isolates, no significant co-localization with Rab7 was observed at any point in time used in this study (**Fig. 4G**). These results imply that maturation of the *C. jejuni* containing vesicles does not always occur independently of Rab7 in the Caco-2 cells. Next, we established that *C. jejuni* ganglioside mimic expression is not the factor

involved in the lack of *C. jejuni* isolates to co-localize with Rab7, an observation that was confirmed using the 2 Δ*cst-II* mutants (GM-) (**Fig. 4H**). Note, at the time frame used to study co-localization of *C. jejuni* with Rab7, *C. jejuni* ganglioside mimic expression was still observed to facilitate cellular binding (**Fig. 4I** and **4J**).

Ganglioside-like LOS enhances the number of *C. jejuni* bacteria co-localizing with Lamp-1. We next determined co-localization with LAMP-1, a marker associated with the fusion between late endosomes and lysosomes [27]. We first tested the isolate 81176 (GM+) that did not co-localize with Rab7 (**Fig. 4A - C**) and the isolate R65 (GM-), that did co-localize with Rab7 (see above). We observed at 2 hours post infection (hpi) that the 81176 isolate co-localized with LAMP-1 (**Fig. 5A - C**), whereas this isolate did not co-localize with Rab7. The GM- isolate R65 did also co-localize with LAMP-1 (**Fig. 5D - F**). We screened all isolates 2 hpi and found that the GM+ isolates GB11, GB19 and 81176 co-localized with LAMP-1 in enhanced numbers compared to the GM- isolates R104, R65 and 9141 (**Fig. 5G**). We also observed that GM+ bacteria tended to cluster together in large compartments that co-localized with LAMP-1 (**Fig. 5A - C**); a characteristic that was not observed for GM- isolates (**Fig. 5D - F**).

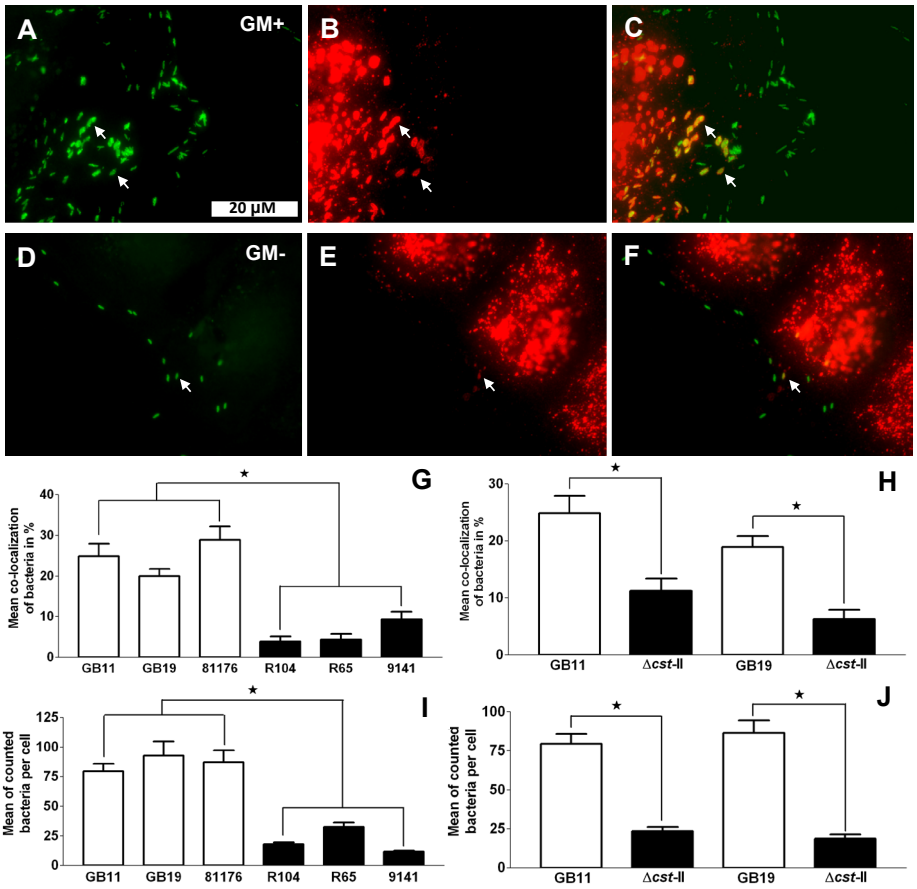

Figure 5 *C. jejuni* ganglioside-like LOS enhances the number of LAMP-1 co-localizing bacteria. Caco-2 cells were incubated with *C. jejuni*, paraformaldehyde fixated at 120 minutes and processed for immuno-fluorescence using, **A** and **D**) anti-*C. jejuni* FITC labelled (green), **B** and **E**) anti-LAMP-1 antibodies (red), **C** and **F**) merging of **A** and **B** and **D** and **E** to visualize LAMP-1 co-localization efficiency of GM+ versus GM- isolates. White arrow shows example of LAMP-1 co-localization. **G**) and **H)** LAMP-1 co-localization of *C. jejuni* was quantified for GM+ (white bars), GM- (black bars), wild type (white bars, GM+) and Δ*cst*-II mutants (Black bars, GM-). Results are shown as the mean percentage of LAMP-1 co-localizing bacteria per cell; error bars show the standard error of the mean of three independent experiments. The difference in co-localization with LAMP-1 between GM+ (white bars) and GM- isolates (black bars) were significant (Mann-Whitney U test, $^{*}p < 0.0001$) and between wild type (white bars, GM+) and Δ*cst*-II mutants (black bars, GM-) (Paired t-test, $^{*}p < 0.0001$). **I)** and **J)** shows the mean of counted *C. jejuni* per cell; error bars show the standard error of the mean of three independent experiments**.** Significant differences in cellular binding were observed between GM+ and GM- wild type isolates (Mann-Whitney U test, $^{*}p < 0.0001$) and between wild type and Δ*cst*-II mutants (Paired t-test, $^{*}p < 0.0001$).

Comparing 2 Δ*cst*-II mutants (GM-) with their wild type isolates (GM+) established that the differences in co-localization with LAMP-1 were dependent on *C. jejuni* ganglioside mimic expression (**Fig. 5H**). At 2 hpi we observed that *C. jejuni* ganglioside mimic expression still promoted cellular binding during the ongoing *C. jejuni* infection of the Caco-2 intestinal epithelial cells (**Fig. 5I and 5J**).

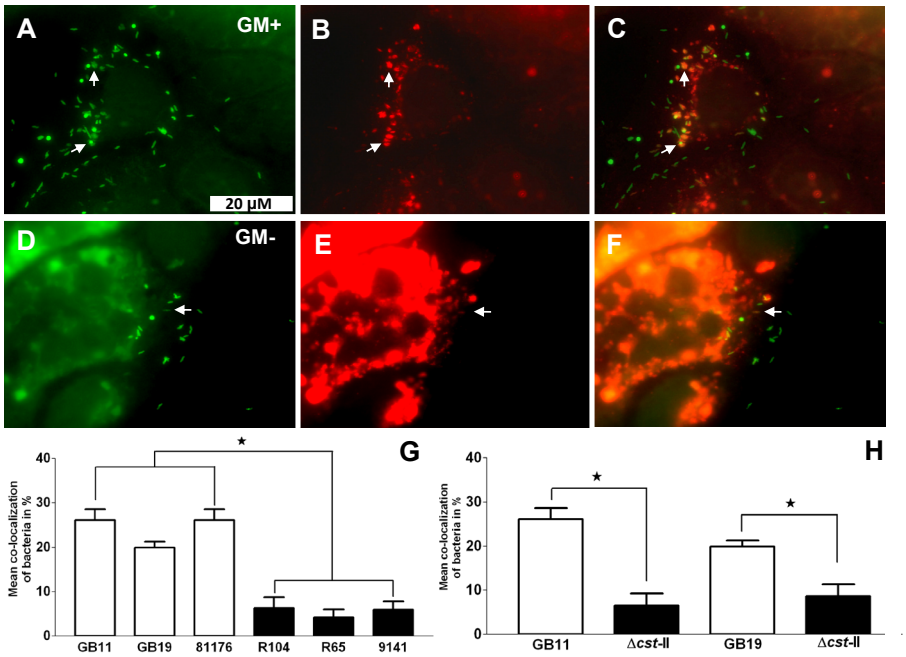

Figure 6 *C. jejuni* – containing vesicles are acidic. Caco-2 cells were incubated with *C. jejuni*, paraformaldehyde fixated at 120 minutes and processed for immuno-fluorescence using, **A** and **D**) anti-*C. jejuni* FITC labelled (green), **B** and **E**) LysoTracker DND-99 (red), **C** and **F**) merging of **A** and **B** and **D** and **E** to visualize the LysoTracker DND-99 co-localization efficiency of GM+ versus GM- isolates. White arrow shows example of co-localization. **G**) and **H)** LysoTracker DND-99 co-localization of *C. jejuni* was quantified for GM+ (white bars), GM- (black bars), wild type (GM+) and Δ*cst*-II mutants (GM-). Results are shown as the mean percentage of co-localizing bacteria per cell; error bars show the standard error of the mean of three independent experiments. The difference in co-localization with LysoTracker DND-99 between GM+ (white bars) and GM- isolates (black bars) were significant (Mann-Whitney U test, $^{*}p < 0.0001$) and between wild type (white bars, GM+) and Δ*cst*-II mutants (black bars, GM-) (Paired t-test, $^{*}p < 0.0001$).

***C. jejuni* – containing vesicles are acidic.** Next, we explored the nature of the *C. jejuni* containing vesicles that co-localized with LAMP-1, by using LysoTracker DND-99, a stain specific for acidic (endo-) lysosomes [28]. At 2 hpi we observed that the GM+ isolate 81176 co-localized in enhanced numbers with LysoTracker DND-99 (**Fig. 6A - C**) compared to the GM- isolate R65 (**Fig. 6D - F**). To confirm this observation we tested the GM+ isolates GB11, GB19 and 81176 and the GM- isolates R104, R65 and 9141 for co-localization with LysoTracker DND-99. We found that the GM+ isolates co-localized in enhanced numbers with LysoTracker DND-99 compared to the GM- isolates ($p < 0.0001$) (**Fig. 6G**). As was observed 2hpi for LAMP-1 staining, LysoTracker DND-99 co-localization revealed that GM+ isolates clustered in large intra-cellular compartments. We established by comparing 2 Δ*cst*-II mutants (GM-) with their wild type isolates (GM+) that the differences in co-localization with LysoTracker DND-99 were dependent on *C. jejuni* ganglioside mimic expression (**Fig. 6H**).

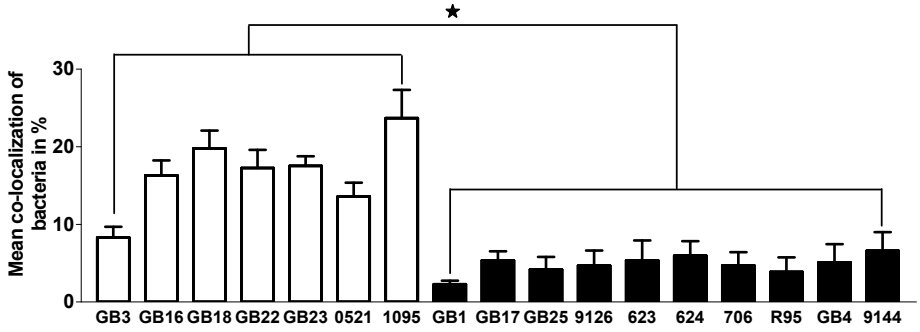

Figure 7 GM+ isolates outnumber GM- isolates in co-localization with LysoTracker DND-99. Caco-2 intestinal epithelial cells were paraformaldehyde fixated at 2 hours post-infection and processed for immuno-fluorescence. The co-localization of C. *jejuni* isolates with LysoTracker DND-99 was quantified for GM+ (white bars) and GM- (black bars) isolates and results are expressed mean co-localization of bacteria in percentage per cell. Error bars show the standard error of the mean of three independent experiments. The difference in co-localization with LysoTracker DND-99 between GM+ versus GM- isolates was significant (Mann-Whitney U test, * $p < 0.0001$).

To investigate if there was any isolate's effect on the acidic nature of the *C. jejuni*-containing vacuole, we extended the LysoTracker DND-99 study with 17 additional *C. jejuni* isolates (7 GM+ and 10 GM- isolates) (**Fig. 7**). We confirmed that the GM+ isolates co-localized significantly more often with LysoTracker DND-99 than the GM- isolates ($p < 0.0001$) (**Fig. 7**). These results suggest that both GM+ and GM- isolates finally reside in acidic degradating endo-lysosomal vesicles. For the GM+ isolates more of these vesicles were counted than for the GM- isolates, a result of increased endocytosis.

***C. jejuni* survival in Caco-2 cells correlates with the efficiency of cellular endocytosis.** A previously published survival assay [3] was used to study whether the enhanced cellular endocytosis by GM+ isolates correlated with increased intra-cellular survival. All isolates (GM+ and GM-) showed approximately a 100 fold decrease in colony forming units (CFU) per ml at 24 hpi (**Fig. 8A** and **8B**), compared to the initial numbers at 4 hpi of Caco-2 cells (**Fig. 8C** and **8D**). Intra-cellular survival calculated in percentage revealed no significant differences between GM+ and GM- isolates; only 1 to 3% survived after 24 hpi from the initially invaded GM+ and GM- isolates detected at 4 hpi. In contrast, the total number of intra-cellular recoverable GM+ isolates, determined as CFU/ml was significantly elevated compared to the GM- isolates ($p < 0.0001$) (**Fig. 8A**). This finding was established when using the two wild type isolates GB11, GB19 (GM+) and their Δ*cst*-II mutants (GM-) (**Fig. 8B**). Complementation of the *cst*-II gene in the GB11Δ*cst*-II mutant restored ganglioside mimic expression and increased survival efficiency to near wild type levels observed with the original GB11 isolate (**Fig. 8E**). The survival assay thus shows that when more *C. jejuni* bacteria enter the Caco-2 cell the more will survive a process strongly dependent on *C. jejuni* ganglioside mimic expression.

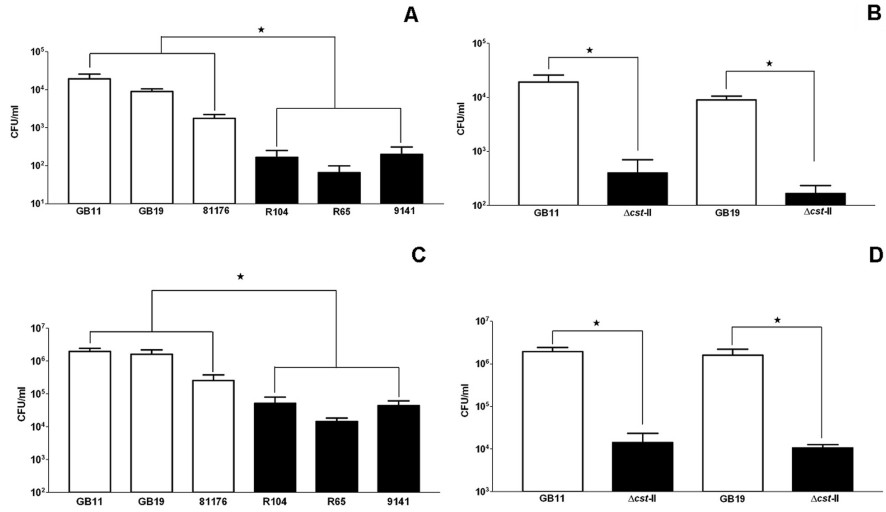

Figure 8 *C. jejuni* survival in Caco-2 intestinal epithelial cells relies on ganglioside-like LOS dependent endocytosis. Survival of *C. jejuni* isolates in Caco-2 intestinal epithelial cells after 24 hours post-infection for **A)** wild type isolates only and **B)** wild type and Δ*cst*-II mutants. Invasion capacity after 4 hours for **C)** wild type isolates only and **D)** wild type and Δ*cst*-II mutants. Survival and intra-cellular recovery are depicted in colony forming units per millilitre (CFU/ml). White bars show the GM+ expressing isolates and the black bars show the GM- expressing isolates. Data are shown as the standard error of the mean of at least three independent experiments. Differences in invasion and survival between wild type isolates only (GM+ and GM-) (Mann-Whitney U test, $^{*}\ p < 0.0001$) and wild type and Δ*cst*-II mutants (Paired t-test, $^{*}\ p < 0.005$) were significant.

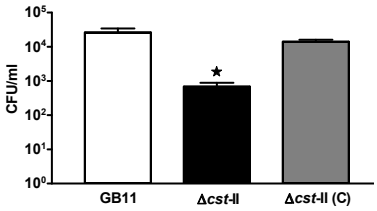

Figure 8E *C. jejuni* numbers of survivng bacteria in the Caco-2 cells depends on ganglioside-like LOS. Complementation of the GB11Δ*cst*-II mutant (Δ*cst*-II (C) restored ganglioside mimic expression and numbers of surviving bacteria to approximately wild type levels. Data are shown as the standard error of the mean of at least three independent experiments performed in duplicate. Differences in survival between wild type (GM+) and Δ*cst*-II mutant (GM-) were significant (Paired t-test, $^{*}\ p < 0.005$) and between Δ*cst*-II mutant (GM-) and Δ*cst*-II complemented mutant Δ*cst*-II (C) (GM+) (Paired t-test, $^{*}\ p < 0.005$).

***C. jejuni* translocation across Caco-2 intestinal epithelial cells is facilitated by ganglioside-like LOS.** *C. jejuni* endocytosis is suggested to be important for epithelial translocation [1, 29]. We hypothesized that ganglioside mimic dependent endocytosis, correlating with increased numbers of surviving bacteria, could be a key factor in the *C. jejuni* translocation process across differentiated Caco-2 cells. Using a Transwell system with differentiated Caco-2 cells revealed that GM+ expressing *C. jejuni* isolates translocated more efficiently after 72 hours compared to their isogenic Δ*cst*-II mutants (GM-), without disrupting the trans-epithelial resistance (TER). On average we sampled from the basolateral side for GB11 and GB19 (GM+), 4.9 x10^4 CFU/ml and 4.4 x10^4 CFU/ml translocating bacteria, respectively (**Fig. 9A**), but for their isogenic Δ*cst*-II mutants (GM-) this was a factor 100 lower, 8.6 x10^2 CFU/ml and 4.3 x10^2, respectively (**Fig. 9A**).

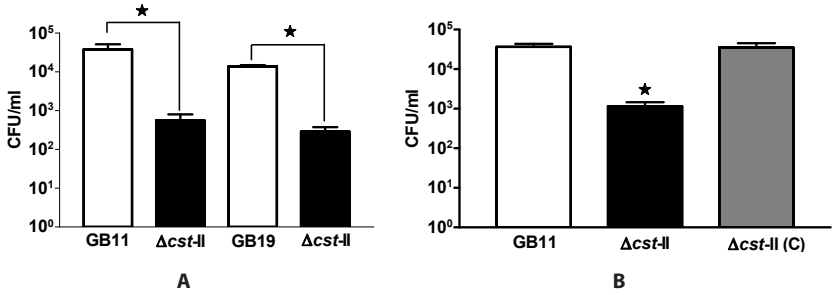

Figure 9 *C. jejuni* translocation across Caco-2 intestinal epithelial cells is facilitated by ganglioside-like LOS. A) Translocation of *C. jejuni* isolates through Caco-2 intestinal epithelial cells was measured as colony forming units (CFU) per ml after 72 hours, using wild type isolates GB11 and GB19 that express ganglioside mimics in comparison with their Δ*cst*-II mutants, GB11Δ*cst*-II and GB19Δ*cst*-II, respectively, lacking ganglioside mimic expression. **B)** Complementation of the GB11Δ*cst*-II mutant (Δ*cst*-II (C) restored ganglioside mimic expression and translocation efficiency to approximately wild type levels. Data are shown as the standard error of the mean of at least three independent experiments performed in duplicate. Differences in translocation between wild type (GM+) and Δ*cst*-II mutant (GM-) were significant (Paired t-test, * $p < 0.005$) and between Δ*cst*-II mutant (GM-) and Δ*cst*-II complemented mutant Δ*cst*-II (C) (GM+) (Paired t-test, * $p < 0.005$).

Restoration of sialylated LOS expression through complementation of the GB11Δ*cst*-II mutant (GM+) confirmed that *C. jejuni* ganglioside mimic expression facilitates intestinal epithelial cell translocation (**Fig. 9B**). In addition, the total number of *C. jejuni* wild type, Δ*cst*-II mutant and complemented Δ*cst*-II mutant (Δ*cst*-II (C)) bacteria that translocated after 72 hpi (**Fig. 9A** and **B**) correlated 1 : 1 with the number of *C. jejuni* bacteria that were recovered from our survival assay (**Fig. 8B** and **8E**).

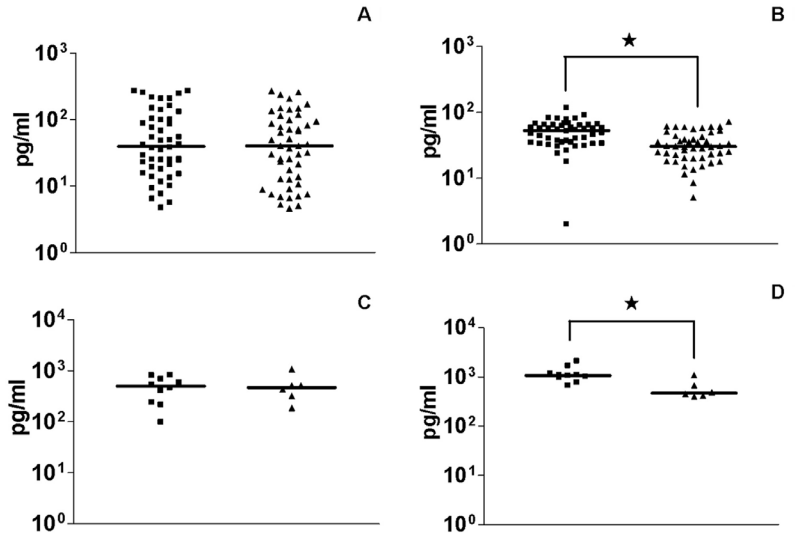

Figure 10 Ganglioside-like LOS expressing *C. jejuni* bacteria elevate the CXCL10 release from intestinal epithelial cells. A) CXCL8 and **B)** CXCL10 secretion from Caco-2 cells infected with 32 *C. jejuni* isolates (Table 1) were analyzed. Scatter plots show the measurements of 3 independent experiments. **C)** CXCL8 and **D)** CXCL10 secretion from a different intestinal epithelial cell line T84, were determined for a subset of the 32 isolates. Blocks represents *C. jejuni* sialylated LOS expressing isolates (GM+) and the triangles represent the *C. jejuni* isolates that lack expression of sialylated LOS (GM-). An asterisk (*) indicates a significant difference in released secretion of the corresponding chemokine from the Caco-2 cells (Mann-Whitney U test, $p < 0.0001$) or the T84 cells (Mann-Whitney U test, $p < 0.0047$).

Ganglioside-like LOS expressing *C. jejuni* bacteria elevate the CXCL10 release from intestinal epithelial cells. To characterize the Caco-2 intestinal epithelial cell response to GM+ and GM- expressing *C. jejuni* isolates (**Supplemental Table 1**), we determined CXCL8 (recruitment of neutrophils) and CXCL10 (recruitment of T cells) release upon infection. CXCL8 secretion by Caco-2 cells following infection by GM+ or GM- isolates was not significantly different (**Fig. 10A**). In contrast, CXCL10 secretion was significantly elevated upon infection by isolates expressing sialylated LOS ($p < 0.0001$) (**Fig. 10B**). To confirm the generality of this finding, a subset of 10 GM+ and 6 GM- isolates was used to test the CXCL8 and CXCL10 secretion using a second intestinal epithelial cell line T84. Using this cell line we confirmed that the CXCL8 secretion was not significantly different, whereas for CXCL10 the secretion was significantly elevated upon infection by GM+ isolates ($p = 0.0047$) (**Fig. 10C** and **10D**).

DISCUSSION

Ganglioside mimic expression by *C. jejuni* has been repetitively shown to correlate with disease complications in man [16, 17, 21], but the pathogenic mechanism(s) behind this process remains obscure. Here in the present study we extended our previous findings [21, 30, 31] aimed

to characterize the role of ganglioside mimic expression in *C. jejuni* pathogenesis and induction of disease complications. Some intra-cellular bacteria have developed mechanisms to avoid delivery to lysosomes in intestinal epithelial cells to be able to survive, replicate, and translocate [26]. Earlier, for the *C. jejuni* isolate 81176 it was shown that this bacterium survives intra-cellular by lysosomal escape [3]. In this study we were able to link ganglioside mimic expression using a wide variety of *C. jejuni* isolates with increased Caco-2 cell endocytosis, but not with lysosomal escape. So far, *C. jejuni* lysosomal escape has only been visualized with endosomal protein markers in non-intestinal epithelial cells [3, 32]. In our study using Caco-2 intestinal epithelial cells we could clearly demonstrate that *C. jejuni* isolates reside in an acidic compartment, which was accompanied by extensive killing of both GM+ and GM- *C. jejuni* bacteria observed after analysis using the Watson and Galan survival assay [3]. Our finding that only a small but equal percentage (1 - 3%) of the *C. jejuni* bacteria (GM+ and GM-) survives 24 hpi suggests that *C. jejuni* survival in the Caco-2 intestinal epithelial cells depends on the number of endocytosed bacteria. A simple explanation for this finding could be that Caco-2 intestinal epithelial cells do not have enough "endosomal capacity" to clear all infecting bacteria. Data showing that *C. jejuni* bacteria are completely eradicated by professional phagocytic cells such as macrophages [3] gives support to this notion. Whether in Caco-2 intestinal epithelial cells the few intra-cellular surviving *C. jejuni* bacteria make use of targeted lysosomal escape or that the endosomal machinery is just simple overloaded disabling bacterial eradication remains to be elucidated.

Our Transwell experiments show that the differentiated Caco-2 cells are an efficient barrier to block the enteric pathogen *C. jejuni*, because after 72 hpi only 1 - 3% of the initial invaded bacteria after 4 hpi is able to translocate across Caco-2 differentiated monolayers. In a previous study it was concluded that *C. jejuni* translocation occurs via transcytosis (apical endocytosis and basolateral exocytosis) [1]. We were here able to confirm this study, since we did not observe a decrease in the TER in our Caco-2 Transwell system, a strong indication for intact tight-junctions [33], during our translocation assay. Next, visualization of the *C. jejuni* endocytosis process with endosomal protein markers imply that *C. jejuni* is transported across the Caco-2 epithelial cells in an endosomal compartment. More importantly, the number of intra-cellular surviving bacteria corresponded perfectly with the number of translocating bacteria in our assays. These data suggests that *C. jejuni* translocation occurs transcellular across differentiated Caco-2 cells, a process found in our study to depend strongly on *C. jejuni* ganglioside-like LOS.

Next, infection of Caco-2 intestinal epithelial cells by ganglioside mimic expressing *C. jejuni* bacteria resulted in an elevated CXCL10 response from intestinal epithelial cells as compared to infection by *C. jejuni* bacteria lacking ganglioside mimics. CXCL10 is a chemoattractant for T cells and correlates with *C. jejuni* invasion [34]. We hypothesize based on our data presented here that ganglioside mimic dependent endocytosis, translocation and elevated CXCL10

release for T-cell recruitment could form the basis for the severe gastro-enteritis phenotype as observed in patients infected with ganglioside mimic expressing *C. jejuni* isolates [17].

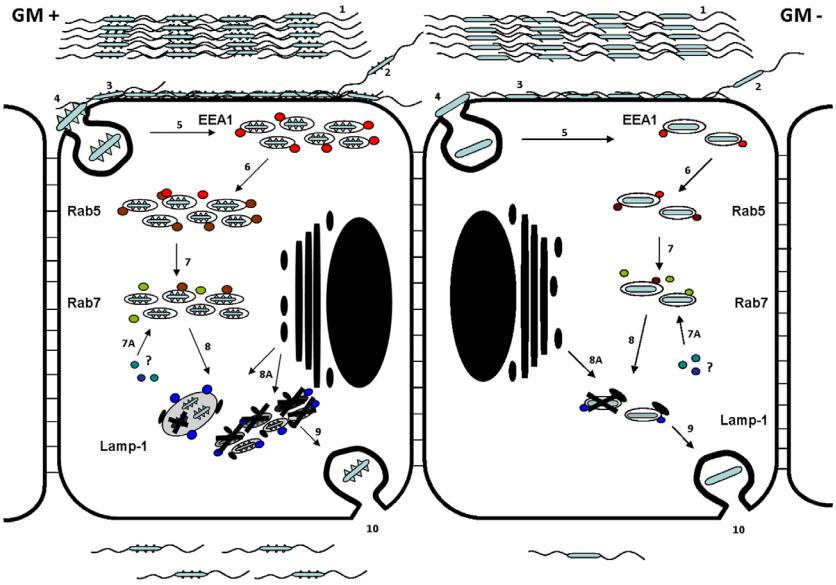

Figure 11 Schematic model for *C. jejuni* ganglioside-like LOS dependent cellular endocytosis, survival and translocation. [1] Equal amounts of *C. jejuni* bacteria that express ganglioside mimics or do not express ganglioside mimics before adherence at an intestinal epithelial cell. [2] The translocation process starts by *C. jejuni* bacteria attaching to the Caco-2 intestinal epithelial cell at the apical surface. [3] *C. jejuni* ganglioside-like LOS is used to bind onto the cell membrane of the Caco-2 intestinal epithelial cell. [3] In the presence of ganglioside-like LOS, *C. jejuni* adherence is a 2 to 3 fold more efficient. [4] The presence of ganglioside-like LOS enhances the cellular entry process (endocytosis) based on studying the early endosomal markers EEA1 and Rab5. [5] Early endosomal antigen 1 (EEA1, red dot) co-localizes to the formed *C. jejuni* containing endosome 30 minutes post-infection. [6] Rab5 (brown dot) is obtained to the *C. jejuni* containing endosome 40 minutes post-infection. [5,6] Increased numbers of ganglioside mimic expressing bacteria are found to co-localize with the endosomal markers EEA1 and Rab5, reflecting increased endocytosis. [7] 40 - 45 minutes post-infection, Rab7 (green dot) will co-localize to some bacteria that do not express ganglioside-like LOS, but most of the *C. jejuni* isolates with or without ganglioside-like LOS will not co-localize with Rab7 within the first two hours of infection. [7A] Maturation of the endosome does not necessarily need Rab7, other factors (Rab GTPases) might be acquired to the *C. jejuni* containing endosome to mature this compartment for lysosomal fusion. [8,8A] Two hours post-infection LAMP-1 (blue dot) co-localizes to the *C. jejuni* containing vesicle and lysosomes (black dot) fuse with the *C. jejuni* containing endosome. During this time *C. jejuni* containing endosomes show fusion when ganglioside-like LOS is expressed, this creates large endosomes with therein multiple bacteria. LAMP-1 and LysoTracker DND-99 co-localization reveals that the *C. jejuni* containing vesicles have strong characteristics of an endo-lysosome. [9] Only 1 - 3% of the invading bacteria survives and are able to translocate at the basolateral surface between 24 - 72 hours post-infection. [10] Translocation occurs more efficient for isolates expressing ganglioside-like LOS compared to isolates lacking ganglioside-like LOS. The numbers of bacteria that survive intra-cellular stands 1:1 to the numbers that are able to translocate.

We conclude that *C. jejuni* translocation across intestinal epithelial cells is facilitated by ganglioside-like LOS, as depicted in a graphical model (**Fig. 11**). As such, LOS sialylation belongs to the repertoire of virulence factors that are used by a subset of *C. jejuni* bacteria to interact with the host epithelium.

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EXPERIMENTAL METHODS

Bacterial strains. 35 clinical *C. jejuni* isolates and the reference isolate 81176 were used in this study (**Supplemental Table 1**). To confirm our data on the wild type isolates we used two previously generated Δ*cst-II* mutants, GB11Δ*cst-II* and GB19Δ*cst-II* and a *cst*-II complemented isolate GB11Δ*cst-II*Δ. The *cst*-II gene encodes a sialyltransferase that transfers sialic acid onto LOS structures [20]. The specific protocols for generation of the Δ*cst*-II mutants and complemented *cst*-II mutant can be found elsewhere [21,22]. To minimize *in vitro* passaging, *C. jejuni* isolates were recovered from the original glycerol stock by culturing on Butzler agar plates (Becton Dickinson, Breda, The Netherlands). The Δ*cst*-II mutants were then grown on generated blood agar plates, supplemented with vancomycin (10 μg/ml) and chloramphenicol (20 μg/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) and the complemented Δ*cst*-IIΔ mutant on erythromycin (0.02 μg/ml) for selection. A last passage on commercial Columbia blood agar plates (Becton Dickinson, Breda, The Netherlands) for wild type, Δ*cst*-II mutants and complemented Δ*cst*-IIΔ mutant was allowed for optimal vitality and equal growth conditions before use. All isolates, mutants and complemented mutant were incubated at 37 ^{0}C in an anaerobic jar under micro-aerophilic conditions using an Anoxamat gas mixer (Mart, Drachten, The Netherlands). Bacterial cells were harvested in pre-warmed 37 ^{0}C Hanks Balanced Salt Solution (HBSS) (Life Technology, Breda, The Netherlands) and densities were adjusted to equal amounts using the optical density (OD) at 600_{nm}. LOS class was determined by PCR, using protocols previously described [21].

Intestinal epithelial cell line. Human intestinal epithelial cells (Caco-2 and T84) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Breda, The Netherlands) and 1% non-essential amino acids (NEAA) (Invitrogen, Breda, The Netherlands). The cells were routinely grown in a 75-cm^2 flask (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) at 37 ^{0}C, 5% CO_2 in a humidified air incubator.

Adhesion to intestinal epithelial cells. Caco-2 cells were grown to 40 - 50% confluence on chamberslides (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). *C. jejuni* was inoculated at a multiplicity of infection (MOI) of 100 onto the chamberslides and between 10 and 15 minutes after inoculation the Caco-2 cells were fixated in 4% formaldehyde HBSS solution at room temperature for 2 hours. Cells were washed three times with HBSS and blocked with 1% fetal bovine serum in HBSS (block buffer). To visualize *C. jejuni* an anti-*C. jejuni* fluorescein (FITC labelled) antibody (Genway, San Diego, California, United States) was 1:100 diluted in block buffer and incubated for 1 hour. Chamberslides were washed 3 times again with HBSS, fixated for 1 min with 70% ethanol and another 1 min with 100% ethanol. The chamber was removed from the slide and the slide was air dried. Slides were mounted with fluorescent mounting medium (Dako, Carpinteria, California, USA). A coverslip was placed on top and slides were analyzed on a XI51 phase contrast fluorescence microscope (Olympus, Leiden,

The Netherlands). Photos were taken with an Olympus XM10 color camera at 1000x magnification and analyzed for adhesion with the Olympus software Cell^F (Olympus). For each isolate, an average of 30 pictures was taken at random fields showing adherence of *C. jejuni* bacteria to Caco-2 cells. Experiments were repeated three times.

Invasion of intestinal epithelial cells. Epithelial cell invasion by *C. jejuni* was determined by growing the intestinal epithelial cells (Caco-2) to confluence for 48 hours at a final density of approximately 5.0 x 10^6 cells per well (Greiner Bio-one, Alphen a/d Rijn, The Netherlands), without allowing them to differentiate. The invasion protocol was performed as previously described [22].

Immunofluorescence. Caco-2 cells were grown to 40 - 50% confluence on chamberslides (Greiner Bio-one) and *C. jejuni* was inoculated with a MOI of 100. Time series were started to detect the optimal co-localization time for the endosomal primary antibody markers EEA-1 (Biosciences BD, Breda, The Nederlands), Rab5 (Santa Cruz, California, United States), Rab7 (Abcam, Cambridge, United states) and LAMP-1 (Abcam, Cambridge, United states). For EEA-1 this was after 30 - 35 min of inoculation for Rab5 35 - 40 min and Rab7 this was 40 - 45 min after inoculation of *C. jejuni* into the chambers. For LAMP-1 the incubation time was 2 hours after inoculation. Prior to visualization of co-localization, chamberslides were washed 2 times with 37 ^{0}C pre-warmed HBSS (Invitrogen) and fixated in 4% formaldehyde HBSS solution at room temperature for 2 hours. Caco-2 cells were washed again 2 times with HBSS. Afterwards the Caco-2 cells were permeabilized for 20 minutes with 0.1% HBSS/Triton X-100 solution, blocked with block buffer 1% fetal bovine serum/ 1% Tween-20/HBSS solution and than incubated for 1 hour with the respective primary antibody (EEA-1, Rab5, Rab7 or LAMP-1) at a 1:100 dilution in block buffer. The appropriate secondary antibodies from the IgG class (H + L), A594 labelled (Molecular Probes) were selected for EEA1, Rab5, Rab7 and LAMP-1. Further preparation and visualization occurred as described in the method section on adherence of intestinal epithelial cells. Chamberslides were screened for co-localization with the different endosomal markers. Experiments were repeated three times. In order to obtain the mean co-localization of bacteria in %, co-localizing bacteria were divided by the bacteria that did not co-localize and multiplied by 100.

Lysotracker DND-99 staining. We used the protocol as described by the manufacturer (Invitrogen, Breda, The Netherlands). Briefly, the stock solution was diluted to a final working concentration of 1mM in DMEM medium containing 10% FBS and 1% NEAA. Caco-2 epithelial cells were pre-incubated with LysoTracker DND-99 for 30 minutes under culture conditions described above. After pre-incubation a *C. jejuni* isolate was added to a well in the chamberslide at a MOI of 100. After 2 hours incubation time Caco-2 cells were washed 3 times with pre-warmed HBSS at 37 ^{0}C to remove excess bacteria and LysoTracker DND-99. Caco-2 cells were fixated in 4% formaldehyde/HBSS solution, washed another 3 times with HBSS, fixated for 1 min with 70% ethanol and another 1 min with 100% ethanol. Further

preparation and visualization occurred as described in the method section on adherence to intestinal epithelial cells.

Intracellular survival. To assay intracellular survival a different culture method was used as described by Watson and Galan [3]. Initially the invasion protocol was followed; after gentamicin treatment the medium was replaced with complete DMEM medium still containing gentamicin (10 µg/ml) (Sigma-Aldrich) without penicillin (Sigma-Aldrich) and streptomycin (Sigma-Aldrich) for overnight incubation. Plating of the infection medium showed that no colony forming units per millilitre (CFU/ml) were present after this treatment. After overnight incubation 3 additional washes with HBSS were performed where after the invasion protocol for plating was followed as previously described [3].

Translocation of *C. jejuni* in a transwell system. Caco-2 cells were seeded onto Transwell filters at 4 x 10^5 cells/filter (5 µm pore size, 1.13 cm2; Costar, Corning Inc. Corning, NY). The Caco-2 cells were allowed to differentiate and formed tight junctions for 19 days [23,24]. *C. jejuni* isolates were added at a MOI of 10 to the apical surface of the Transwell filter. After 72 hours 100 µl samples were taken from the apical and basolateral surface. Serial dilutions (1:10) were made and plated on Columbia blood agar plates (Becton Dickinson). Plates were incubated for 24 hours at 37 ^{0}C in an anaerobic jar under micro-aerobic conditions using an Anoxamat gas mixer (Mart). Colonies were counted and the CFU/ml was calculated.

CXCL8 and CXCL10 measurement. For CXCL8 and CXCL10 measurements 96-well flat bottomed plates (Nunc) were coated with 3ng of rabbit anti-human CXCL8 or CXCL10 (Endogen, Cambridge, Mass) per well. Overnight culture supernatants from confluent grown Caco-2 cells in 6-well plates infected at a MOI of 100 with wild type isolates (Table 1) were diluted 1:1 in phosphate buffered saline (PBS) supplemented with 0.1% Tween-20 and 3mg of bovine serum albumin/ml. CXCL8 and CXCL10 were detected and quantified against a calibration line with a biotin coupled detection antibody (0.5mg/ml). The assay was developed with avidin-peroxidase (500 mg/ml) (Gibco-BRL) and TMB (3,3',5,5'-tetramethylbenzidine; Sigma) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using Instat software (version 2.05a; GraphPad Software, San Diego, CA). Log transformation was used to equalize variances. Differences between GM+ isolates and GM- isolates, were tested for significance with a Mann-Whitney U test, since column statistics showed that the Gaussian distribution was unequal for the isolates. A two-tailed value with $p < 0.05$ indicated statistical significance. Statistical analysis for differences between wild-type and knockout mutant isolates were tested for significance with a Paired t-test.

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Supplemental Table 1

Isolate [1]	Presence LBGES [2]	Ganglioside mimic [3]	Isolate [1]	Presence LBGES [2]	Ganglioside mimic [3]
GB1	+	none	E9141	-	ND
GB3	+	GM1, GD1a	E9144	-	ND
GB4	-	none	E9146	-	ND
GB11	+	GM1, GD1a	E990521	+	ND
GB16	+	GD1c	E9126	+	ND
GB17	+	GA1, GM1b, GD1c	E98-1095	+	ND
GB18	+	GM1, GD1a	E98-623	-	none
GB19	+	GD1c	E98-624	-	none
GB22	+	GM1a, GD1a	E98-706	-	none
GB23	+	GM2	E98-1033	+	GM1
GB25	+	GA1, GM1b, GD1c	E98-652	+	GM1a, GQ1b
GB29	+	none	E98-682	+	GM1a. GQ1b
81176	+	GM2/GM3	E98-1087	+	GM1a
11168	+	GM1/GM2	R65	-	ND
R3	-	none	R67	-	ND
R33	-	ND	R95	-	GA1
R37	-	none	R104	-	ND
R61	-	ND	R109	+	GM1a

Supplemental Table 1 *C. jejuni* isolates, presence of genes needed for sialic acid biosynthesis and ganglioside mimics expressed. [1] Isolates used to study co-localization of *C. jejuni* with LysoTracker DND-99 and chemokine secretion. GB indicates Guillain-Barré, R and E indicate enteritis-associated isolates. [2] PCR results, scoring the presence +, or absence - of the LOS Biosynthesis Genes Enabling Sialylation (LBGES). [3] Ganglioside mimics (GM) detected with mass spectrometry; GA1 indicates asialo-gangliosides mimic, "none" indicates no ganglioside mimics detected; ND indicates not determined. For the other isolates GM structures were determined by rapid mass spectrometry screening or by antibody probing (652, 682, 1033, 1087, R95, R109). Note presence of LBGES doesn't always mean ganglioside mimic expression due to phase variation.

Chapter 5

Correlation between genotypic diversity, lipo-oligosaccharide gene locus class variation, and Caco-2 cell invasion potential of *Campylobacter jejuni* isolates from chicken meat and humans: contribution to virulotyping

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ABSTRACT

Significant interest in studying the lipo-oligosaccharide (LOS) of *Campylobacter jejuni* has stemmed from its potential role in post-infection paralytic disorders. In this study we present the results of PCR screening of five LOS locus classes (A, B, C, D, and E) for a collection of 116 *C. jejuni* isolates from chicken meat (n = 76) and sporadic human cases of diarrhea (n = 40). We correlated LOS classes with clonal complexes (CC) assigned by multilocus sequence typing (MLST). Finally, we evaluated the invasion potential of a panel of 52 of these *C. jejuni* isolates for Caco-2 cells. PCR screening showed that 87.1% (101/116) of isolates could be assigned to LOS class A, B, C, D, or E. Concordance between LOS classes and certain MLST CC was revealed. The majority (85.7% [24/28]) of *C. jejuni* isolates grouped in CC-21 were shown to express LOS locus class C. The invasion potential of *C. jejuni* isolates possessing sialylated LOS (n = 29; classes A, B, and C) for Caco-2 cells was significantly higher ($p < 0.0001$) than that of *C. jejuni* isolates with non-sialylated LOS (n = 23; classes D and E). There was no significant difference in invasiveness between chicken meat and human isolates. However, *C. jejuni* isolates assigned to CC-206 (correlated with LOS class B) or CC-21 (correlated with LOS class C) showed statistically significantly higher levels of invasion than isolates from other CC. Correlation between LOS classes and CC was further confirmed by pulsed-field gel electrophoresis. The present study reveals a correlation between genotypic diversity and LOS locus classes of *C. jejuni*. We showed that simple PCR screening for *C. jejuni* LOS classes could reliably predict certain MLST CC and add to the interpretation of molecular-typing results. Our study corroborates that sialylation of LOS is advantageous for *C. jejuni* fitness and virulence in different hosts. The modulation of cell surface carbohydrate structure could enhance the ability of *C. jejuni* to adapt to or survive in a host.

INTRODUCTION

Campylobacter jejuni is an important human enteric pathogen worldwide [1-3]. Infected humans exhibit a range of clinical spectra, from mild, watery diarrhea to severe inflammatory diarrhea [4]. Factors influencing the virulence of *C. jejuni* include motility, chemotaxis and adherence, invasion of intestinal cells, intracellular survival, and toxin production [4-6]. Besides its role in human enteric illnesses, *C. jejuni* is a predominant infectious trigger of acute post-infectious neuropathies, such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) [7]. Significant interest in studying the structure and biosynthesis of the core lipo-oligosaccharide (LOS) of *C. jejuni* has resulted from its potential role in these paralytic disorders. Many studies have now provided convincing evidence that molecular mimicry between *C. jejuni* LOS and gangliosides in human peripheral nerve tissue plays an important causal role in the pathogenesis of GBS/MFS [8-11].

Initial comparative studies of *C. jejuni* LOS structure and the corresponding DNA sequences of the LOS biosynthesis loci identified eight different LOS locus classes. Three of these classes, A, B, and C, harbor sialyltransferase genes involved in incorporating sialic acid into the LOS [12]. Sialylation of the LOS core was found to be associated with ganglioside mimicry and also to affect immunogenicity and serum resistance [8]. Recently, Parker *et al*. [13] identified 11 additional LOS classes on the basis of the sequence at the LOS biosynthesis locus. Their investigation also suggested that the LOS loci of *C. jejuni* strains are hot spots for genetic exchange, which can lead to mosaicism.

Despite evidence on locus variation within *C. jejuni* LOS classes, PCR-based screening of a collection of 123 clinical and environmental strains showed that almost 60% of *C. jejuni* strains belong to class A, B, or C [12]. Additionally, Godschalk *et al*. [9] found that 53% (9/17) of GBS-associated *C. jejuni* strains possessed LOS of class A, while 64% (35/55) of the non-GBS-associated isolates possessed LOS of class A, B, or C, and 62% (13/21) of enteritis-associated *Campylobacter* strains expressed LOS of class A, B, or C, as well. This relative representation of sialylated LOS classes A, B, and C was hypothesized to be advantageous for *C. jejuni* in the colonization and infection of various hosts [12, 14]. Recently, Louwen *et al*. [15] demonstrated that *C. jejuni* strains possessing sialylated LOS (class A, B, or C) invade Caco-2 cells significantly better than non-sialylated strains (with class D or E). Knockout mutagenesis of the LOS sialyltransferase Cst-II in three *C. jejuni* strains revealed a significant reduction in the invasion potentials of the mutant strains [15]. The possible role of LOS in adhesion and invasion was previously highlighted in the work of Perera *et al*. [16] and Kanipes *et al*. [17], where a *C. jejuni* waaF mutant strain showed significant reductions in levels of adherence to and invasion of INT-407 cells.

LOS class diversity in *C. jejuni* strains isolated from chicken meat, an important source of human *Campylobacteriosis* [1, 3, 18], has hardly been studied at all. In addition, the role of LOS class variation in the invasion potential of *C. jejuni* strains from chicken meat still needs to be

explored. The epidemiological relevance of *C. jejuni* LOS gene screening can be further elaborated by correlating its results with results from other molecular-typing tools (e.g., multilocus sequence typing [MLST] and pulsed-field gel electrophoresis [PFGE]). In the present study, we screened a diverse collection of *C. jejuni* isolates, from consumer-packaged chicken meats and from sporadic human cases of diarrhea, by PCR for five LOS classes (A, B, C, D, and E). Then we correlated the LOS classes assigned by PCR screening with the genotypes assigned by PFGE and MLST. Finally, we tested the invasion potentials of a representative subset of *C. jejuni* isolates in relation to their LOS classes and genotypic diversity.

RESULTS

LOS locus class diversity. The results presented in **Supplemental Table 1** indicate that 87.9% (102/116) of the *C. jejuni* isolates characterized in this study could be assigned to one of the five LOS locus classes (A to E) screened by the class-specific PCR. *C. jejuni* isolates of LOS class A were significantly underrepresented ($p < 0.05$) compared to other classes, while isolates harboring LOS classes C and B represented, together, almost half (48.3% [56/116]) of the total number of isolates screened. LOS classes B and E were significantly ($p < 0.05$) more frequently represented among *C. jejuni* isolates from humans than among those from chicken meat, amounting to 30% (12/40) and 25% (10/40) of the screened enteritis isolates, respectively.

Genotypic concordance between chicken meat and human isolates. MLST identified 34 STs among the 76 *C. jejuni* isolates from chicken meat, with 15.8% (12/76) of the isolates identified as ST-50 (**Supplemental Table 1**). Overrepresentation of CC-21 was evident; amounting to one-quarter of all chicken meat isolates (**Supplemental Table 2**). *C. jejuni* isolates from human enteritis cases (n = 40) were assigned to 27 STs; 62.9% (17/27) of these STs were singletons, while 4 and 3 isolates were assigned to ST-354 and ST-137, respectively (**Supplemental Table 1**). In agreement with the results for chicken meat isolates, CC-21 was slightly more frequently represented than other clonal complexes in *C. jejuni* isolates of human origin. *C. jejuni* isolates assigned to CC-45, CC-206, and CC-443 were found comparably frequently in the chicken and human collections (**Supplemental Table 2**). However, CC-257 was well represented in *C. jejuni* isolates from chicken meat but was not recorded for isolates of human origin (**Supplemental Table 2**).

Eleven STs were featured in both chicken meat and human *C. jejuni* isolates. One of these, ST-3546, is a novel ST, first reported in this Belgian collection; it was isolated from chicken meat in April 2007 and again from a human diarrheal sample in June 2007 (**Supplemental Table 1**). Other STs found in both human and chicken samples were ST-354, -19, -443, -572, -42, -48, -50, -53, -122, and -775. Thus, in total, 17 of the 40 (42.5%) *C. jejuni* isolates from human diarrheal samples were found to share STs with isolates from chicken meat preparations.

MLST and PFGE typing versus LOS PCR classes. The results presented in **Fig. 1** show a concordance between the *C. jejuni* LOS locus classes assigned by PCR and certain MLST clonal complexes. Of note, a majority (85.7% [24/28]) of *C. jejuni* isolates assigned to CC-21 were found to express LOS locus class C. The correlation between LOS class C and CC-21 was evident in both the human and the chicken collection (**Supplemental Table 1**). LOS class B was found in eight different clonal complexes, most frequently in CC-206 (34.6% [9/24]) (**Supplemental Table 1**; **Fig. 1**). In addition, 78.5% (11/14) of *C. jejuni* isolates in CC-45 expressed LOS class E, and 88.9% (8/9) of CC-354 isolates matched PCR assignment to LOS class D (**Supplemental Table 1**). The correlations between LOS class D and CC-354 and between LOS class E and CC-45 were evident in both the human and chicken collections.

We were interested in obtaining further insight into the correlation between sequence typing and LOS PCR assignment by using PFGE typing. **Fig. 2** shows that the correlation between sequence typing results and LOS PCR classifications can be further elaborated by PFGE. For example, and as indicated by MLST (**Supplemental Table 1**), *C. jejuni* isolates assigned to LOS class B were relatively diverse; they were grouped by PFGE into two main clusters delineated by a 60% band similarity cutoff (**Fig. 2.I**).

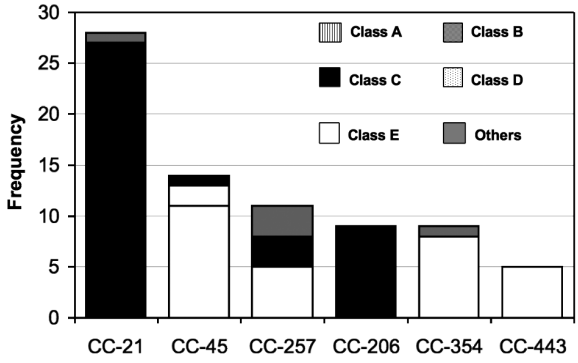


Figure 1 Frequency distribution of *C. jejuni* LOS classes in correlation with selected MLST clonal complexes found for both chicken meat and human isolates.

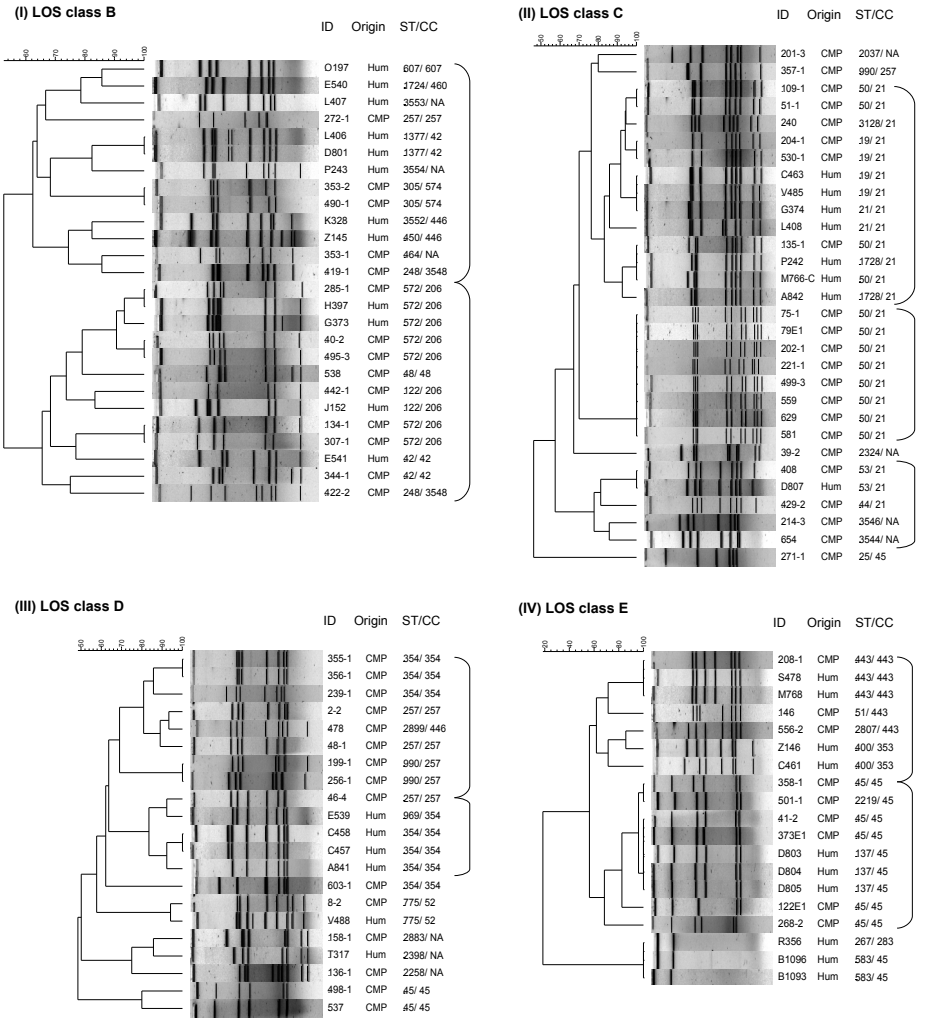


Figure 2 PFGE dendrogram based on band patterns of SmaI-digested DNA from *C. jejuni* isolates in relation to their LOS assignments. PFGE clusters are marked by curved arcs and presented in correlation with strain identification (ID) numbers, origins (human [Hum] or chicken meat preparation [CMP] samples), and STs and clonal complexes (CC) by MLST.

On the other hand, LOS class C isolates were grouped in three very well correlated clusters, among which was a cluster of eight isolates with identical band patterns (**Fig. 2.II**). These eight isolates were sampled over six different months and originated from four different companies and various slaughter batches (**Supplemental Table 1**).

Invasion potential in relation to LOS class, strain source, and genotypic diversity. Fifty-one of the 52 *C. jejuni* isolates tested for invasion potential were able to be internalized into Caco-2 cells; the invasion percentages of these isolates ranged from 0.0002% to 0.26% (**Supplemental Table 3**). Thirteen of the 14 *C. jejuni* isolates for which 0.1% of the inoculum was internalized expressed sialylated LOS classes (A, B, or C). CC-21, followed by CC-206, was the most frequently represented clonal complex among these 14 isolates (**Supplemental Table 3**). There was no significant correlation (*p*, 0.381 by negative binomial regression analysis) between the source of isolates (human or chicken) and the level of invasion of Caco-2 cells (**Fig. 3A**).

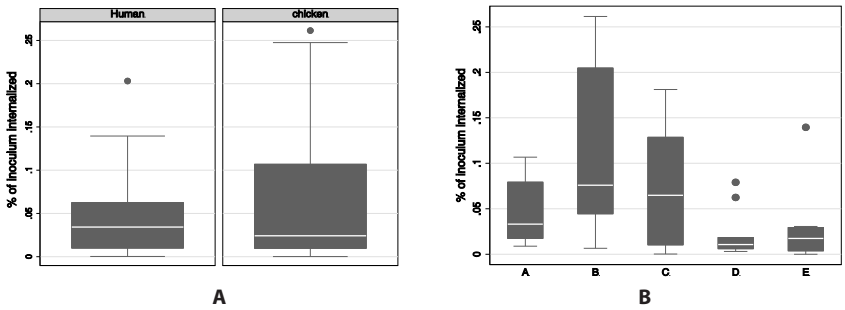
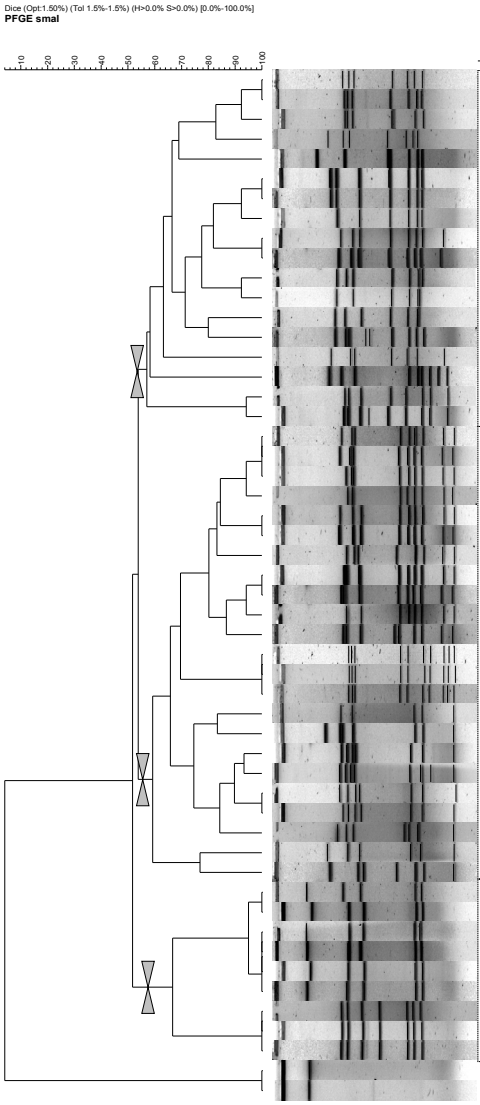


Figure 3 Differences in the abilities of *C. jejuni* strains (n = 52) to invade Caco-2 cells in relation to the sources of the strains (**A**) and their LOS classes (**B**). The lines inside the boxes denote the median percentages of internalization of the inocula, and the upper and lower limits of the error bars signify the 75th and 25th percentiles, respectively. The circles above the boxes represent strains with percentages of internalization higher than the 90th percentile.

The invasion potential of *C. jejuni* isolates of LOS class B was significantly higher than that for other classes (coefficient, 1.66; $p < 0.0001$), followed by *C. jejuni* isolates of LOS class C (coefficient, 1.28; $p < 0.005$) (**Fig. 3B**). Thus, the invasion potential of *C. jejuni* isolates with sialylated LOS (n = 29; classes A, B, and C) was significantly higher ($p = 0.002$) than that of *C. jejuni* isolates with non-sialylated LOS (n = 23; classes D and E).



ID	Origin	MLST ST/CC	LOS	% inoculum internalized	PFGE cluster
146	CMP	51/ 443	E	< 0.01	P1
355-1	CMP	354/ 354	D	< 0.01	
490-1	CMP	305/ 574	B	>= 0.1%	
239-1	CMP	354/ 354	D	>= 0.01% and < 0.1	
603-1	CMP	354/ 354	D	< 0.01	
C458	Hum	354/ 354	D	>= 0.01% and < 0.1	
C457	Hum	354/ 354	D	>= 0.01% and < 0.1	
A841	Hum	354/ 354	D	>= 0.01% and < 0.1	
408	CMP	53/ 21	C	>= 0.1%	
D807	Hum	53/ 21	C	>= 0.01% and < 0.1	
2-2	CMP	257/ 257	D	>= 0.01% and < 0.1	
272-2	CMP	257/ 257	A	>= 0.01% and < 0.1	
46-4	CMP	257/ 257	D	< 0.01	
L406	Hum	1377/ 42	B	>= 0.01% and < 0.1	
353-1	CMP	464/ NA	B	>= 0.1%	
157-1	CMP	704/ NA	A	>= 0.01% and < 0.1	
8-2	CMP	775/ 52	D	>= 0.01% and < 0.1	
V488	Hum	775/ 52	D	>= 0.01% and < 0.1	
135-1	CMP	50/ 21	C	>= 0.1%	P2
M766-C	Hum	50/ 21	C	< 0.01	
P242	Hum	1728/ 21	C	>= 0.01% and < 0.1	
187-1	CMP	3550/ 21	A	>= 0.01% and < 0.1	
501-3	CMP	883/ 21	A	>= 0.1%	
R357	Hum	262/ 21	A	>= 0.01% and < 0.1	
344-1	CMP	42/ 42	B	>= 0.1%	
C463	Hum	19/ 21	C	< 0.01	
G374	Hum	21/ 21	C	>= 0.1%	
530-1	CMP	19/ 21	C	>= 0.01% and < 0.1	
L408	Hum	21/ 21	C	>= 0.1%	
79E1	CMP	50/ 21	C	>= 0.1%	
499-3	CMP	50/ 21	C	>= 0.1%	
559	CMP	50/ 21	C	< 0.01	
442-1	CMP	122/ 206	B	>= 0.1%	
J152	Hum	122/ 206	B	>= 0.01% and < 0.1	
H397	Hum	572/ 206	B	>= 0.1%	
G373	Hum	572/ 206	B	>= 0.01% and < 0.1	
285-1	CMP	572/ 206	B	>= 0.1%	
495-3	CMP	572/ 206	B	>= 0.01% and < 0.1	
199-1	CMP	990/ 257	D	< 0.01	
307-1	CMP	572/ 206	B	>= 0.01% and < 0.1	
E541	Hum	42/ 42	B	< 0.01	
358-1	CMP	45/ 45	E	>= 0.01% and < 0.1	P3
501-1	CMP	2219/ 45	E	>= 0.01% and < 0.1	
41-2	CMP	45/ 45	E	< 0.01	
373E1	CMP	45/ 45	E	>= 0.01% and < 0.1	
D803	Hum	137/ 45	E	>= 0.1%	
D805	Hum	137/ 45	E	< 0.01	
208-1	CMP	443/ 443	E	>= 0.01% and < 0.1	
S478	Hum	443/ 443	E	>= 0.01% and < 0.1	
M768	Hum	443/ 443	E	>= 0.01% and < 0.1	
R356	Hum	267/ 283	E	>= 0.01% and < 0.1	
B1096	Hum	583/ 45	E	< 0.01	

Figure 4 PFGE dendrogram based on SmaI-digested DNA from 52 *C. jejuni* isolates characterized for their potential to invade Caco-2 cells. PFGE clusters (P1, P2, and P3) are grouped in boxes with dashed outlines and are presented in correlation with strain identification (ID) numbers, origins (human [Hum] or chicken meat preparation [CMP] samples), STs and clonal complexes (CC) by MLST, LOS classes, and percentages of inocula internalized in Caco-2 cells.

Further characterization of the 52 isolates included in the invasion assay was based on PFGE typing. **Fig. 4** shows that 50 of these isolates (the other 2 were not restricted by SmaI) can be assigned to three PFGE clusters (P1, P2, and P3). Twenty-two of the 23 *C. jejuni* isolates in PFGE cluster P2 were assigned to the three sialylated LOS classes (**Fig. 4**). Of note, 10 of the 14 isolates for which 0.1% of the inoculum was internalized by Caco-2 cells (**Supplemental Table 3**) were in PFGE cluster P2, regardless of their origins.

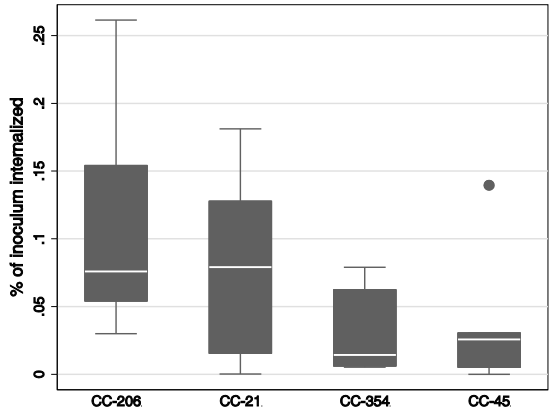


Figure 5 Differences in invasion potential among *C. jejuni* isolates from the four main clonal complexes found in both chicken and human isolates. The lines inside the boxes denote the median percentages of internalization of the inocula, and the upper and lower limits of the error bars signify the 75th and 25th percentiles, respectively. The circle above the CC-45 box represents a strain with an internalization level higher than the 90th percentile.

Fig. 5 shows the differences among the invasion potentials of *C. jejuni* isolates assigned to the four main clonal complexes found in the chicken meat and human collections: CC-21, CC-45, CC-206, and CC-354 (**Supplemental Table 2**). The invasion potential of *C. jejuni* isolates assigned to CC-45 was significantly lower ($p < 0.05$) than those for other clonal complexes. There was no significant difference between CC-206 and CC-21 isolates ($p > 0.05$), but the invasion level of *C. jejuni* isolates in each of CC-206 and CC-21 was significantly higher ($p < 0.05$) than those of isolates in both CC-354 and CC-45. In the comparison between clonal complexes, the number of isolates per clonal complex was selected in proportion to its frequency (**Supplemental Table 2**) and in view of LOS PCR results (**Supplemental Table 1**). For example, 15 of the 28 *C. jejuni* isolates in CC-21 were assayed for invasion and distributed as 12 of the 24 isolates identified with LOS class C plus the 3 isolates identified with LOS class A (**Supplemental Table 3**).

DISCUSSION

The present study adds to the limited knowledge about LOS class diversity in *C. jejuni* isolates from chicken meat and about the invasion potential of such isolates compared to those from human diarrheal samples. In the present study, PCR screening targeted only five specific classes, despite recent increases in the number of LOS locus classes [13]. Nevertheless, a majority (87.1% [101/116]) of the isolates screened was member of these five classes, and 52.6% of *C. jejuni* isolates from Belgian chicken meat and human diarrheal samples possessed sialylated LOS (class A, B, or C).

It is estimated that 0.3 in 1,000 to 1 in 1,000 *Campylobacter* infections leads to GBS. In addition, epidemiological studies have indicated that a *C. jejuni* infection precedes GBS in 20 to 50% of cases in Europe, North and South America, Japan, and Australia [19-20]. *C. jejuni* strains expressing class A and B LOS loci have been associated with post-infectious neuropathy [9]. In the present study, these two classes together were found in almost one-quarter of all chicken meat isolates (**Supplemental Table 1**): 5.3% (4/76) and 18.4% (14/76) of *C. jejuni* isolates from the chicken meat collection were found to express LOS classes A and B, respectively. In a previous study, we showed that the prevalence of *Campylobacter spp*. in Belgian chicken meat preparations, including isolates used in the present study, is around 60% [21]. The high prevalence of *Campylobacter* spp. in chicken meat, combined with the fact that a substantial subset of the *C. jejuni* isolates characterized in this study possess neuropathy-associated LOS, can be regarded as a worrying signal. Therefore, strategies to control *Campylobacter* contamination of chicken meat might reduce the morbidity due to GBS, in addition to reducing the level of *Campylobacter*-related human enteric illnesses.

Human isolates included in the present study were isolated between May and September 2007. Epidemiological evidence from many countries [22-23] suggests that human *Campylobacter*iosis tends to increase during this period of the year. Using MLST, we showed that *C. jejuni* strains isolated from human diarrheal samples during this period exhibit considerable genetic overlap (42.5% [17/40] of human isolates) with isolates from the chicken meat population. In addition, the distribution of MLST clonal complexes showed good concordance between chicken and human isolates (**Supplemental Table 2**); however, CC-21 was more frequently represented than other clonal complexes in both. CC-21 is the largest complex in the general population structure of *C. jejuni* [24]; it is widespread in multiple hosts and has previously been reported to be associated with infections of humans and with livestock and environmental sources, such as chicken, cattle, contaminated milk, and water [24-25]. Molecular epidemiological evidence suggests that this clonal complex is associated with environmental and food-borne transmission [25-26]. Considering the possible epidemiological significance of CC-21, Best *et al*. [27] described a single-nucleotide polymorphism analysis assay enabling rapid strain profiling for CC-21. In the present study, we showed that PCR screening of *C. jejuni* LOS class C could correctly predict CC-21 for 86% of isolates screened (**Supplemental Table 1**). Additionally,

comparable correlations were evident in other LOS class-MLST clonal complex combinations (**Fig. 1**). Thus, PCR screening for *C. jejuni* LOS classes could be of value in population structure studies, especially for elaborating the clonal relationships between *C. jejuni* isolates.

The *C. jejuni* isolates included in this study were selected in such a way as to generate an epidemiologically diverse collection, by including isolates of human and chicken origins, cultured over a period of 10 months. Moreover, the chicken meat collection contained isolates from five different producers and from a variety of processing batches (**Supplemental Table 1**). Thus, for example, the correlation between the dominant clonal complex CC-21 and LOS locus class C (**Fig. 1**) is unlikely to be due to bias or chance. Parker *et al*. [12] indicated that LOS class C was detected in *C. jejuni* isolates from all sources, based on PCR screening of a collection of 123 clinical and environmental strains. In addition, Müller *et al*. [28] found that most *C. jejuni* isolates from human and turkey sources express primarily LOS class C. These studies, in addition to our PCR screening and MLST data, suggest a possible role of LOS class C in the evolution of the widely spread clonal complex CC-21 that might be of particular importance in the poultry meat-related transmission of *C. jejuni* to humans. The correlation between *C. jejuni* with sialylated LOS class C and the ecologically diverse CC-21 could be an example of an adaptive strategy used by *C. jejuni* to modulate cell surface carbohydrate structures in order to better survive in a given host species. However, further screening studies are needed to confirm our hypothesis regarding such presumed correlations.

The correlation between certain MLST clonal complexes and LOS PCR assignment was further elaborated using PFGE. *C. jejuni* isolates of LOS classes A, B, and C were grouped into one PFGE cluster (P2) (**Fig. 4**), indicating a phylogenetic correlation between isolates harboring these sialylated classes. In fact, our results (**Fig. 4**) show that *C. jejuni* LOS classes A and C were actually sharing the same MLST clonal complex (CC-21). Recombination between locus class C and class A can occur between regions of homologues that flank these LOS biosynthesis loci and has been reported previously for *C. jejuni* strain GB11 [29]. Moreover, it is believed that LOS locus class B could be an evolutionary intermediate between classes A and C [29], which could explain our finding of close phylogenetic correlation between *C. jejuni* isolates with LOS class B and those with classes A and C. Thus, the phylogenetic correlation between LOS classes A, B, and C can be attributed to the nature of the LOS loci of *C. jejuni* as hot spots for genetic exchange.

The results from our invasion assays support the growing hypothesis that the enhanced invasiveness of *C. jejuni* strains with sialylated LOS could contribute to post-infectious complications. Perera *et al*. [16] previously showed that the presence of intact LOS is vital for *C. jejuni* adherence to and invasion of INT-407 cells. In addition, our results confirm the recent finding by Louwen *et al*. [15] that *C. jejuni* isolates with sialylated LOS exhibit a higher invasion potential than *C. jejuni* isolates with non-sialylated LOS (classes D and E). Their conclusion was based on *C. jejuni* strains isolated only from human patients with enteritis and GBS, whereas in the present study we extend the same conclusion to *C. jejuni* isolates from ready-to-cook chicken

meat. Of note, we used the same invasion assay protocol as that used by Louwen *et al*. [15], in order to ensure a valid comparison of their and our results.

In the present study, no significant differences in the invasion phenotype were found between *C. jejuni* isolates from patients with diarrhea and *C. jejuni* isolates from chicken meat meant for human consumption. Previous studies correlating invasion phenotypes with the sources of isolates provided contradictory findings; indeed, many of these studies concluded that the invasiveness of clinical strains is higher than that of strains isolated from poultry [30-34]. However, some studies indicate no difference in invasion, or in adhesion, between *C. jejuni* isolates from human and poultry sources [35-36]. Nevertheless, the ability of *C. jejuni* to invade epithelial cells in vitro is recognized as being strain dependent [37-39]. Our results support such a concept to a certain extent; for example, the average invasion potential of *C. jejuni* isolates with sialylated LOS of class B was significantly higher than that for other classes (**Fig. 3B**). However, some of these isolates still exhibit variant invasion phenotypes (**Supplemental Table 3**). On the other hand, the majority of *C. jejuni* isolates with non-sialylated LOS (classes D and E) were associated with a relatively lower invasion potential than strains expressing sialylated LOS classes (**Fig. 3B**). Among *C. jejuni* isolates expressing LOS class D, human isolates showed higher invasion levels (**Supplemental Table 3**) than chicken meat isolates, despite sharing the same ST. Presumably a host adaptation effect was behind this finding.

Invasiveness results from the interplay of numerous bacterial and host factors. PCR screening of seven virulence-associated genes indicated the presence of *ceuE*, *cadF*, *ciaB*, *pldA*, *cdtA*, *cdtB*, and *cdtC* in all strains (data not shown). In addition, we showed that isolates with a common genotypic profile, as identified by MLST clonal complexes and PFGE similarity clusters, might have common in vitro virulence characteristics as well (**Fig. 4**); *C. jejuni* isolates of CC-21 and CC-206 were associated with a high invasion potential, while isolates of CC-45 were less invasive (**Fig. 4**). Clearly there are considerable discrepancies between studies attempting to correlate invasiveness, or other virulence attributes, with certain genotypes of *C. jejuni*. The lack of a common nomenclature for genotype assignment makes it difficult to use these studies to establish a correlation between typing data for *C. jejuni* and a selected virulence trait. Studies using MLST could be of value in solving such dilemma, since the technique uses a robust standard numerical assignment of STs and clonal complexes. Recently, Fearnley and colleagues [30] studied the invasion of INT-407 cells by 113 *C. jejuni* strains. They found that four of five "hyperinvasive" *C. jejuni* strains were associated with CC-21, but their study did not identify a correlation between MLST clonal complexes and a specific pattern of invasion. Alternatively, Hänel *et al*. [40] identified an association between the PFGE genotypes of 17 *Campylobacter* isolates from turkeys and their invasion phenotypes in Caco-2 cells. It should be noted that in our study, the strains selected for the study of invasiveness were found to be inherently associated with certain LOS classes (**Fig. 2**). However, this association seems to be a function of a biological correlation between *C. jejuni* with sialylated/non-sialylated LOS classes and certain genotypes. To the best of our knowledge, the present study is the first to

highlight such a possible biological correlation, and we show an evident impact of such a correlation on the invasion potential of *C. jejuni* strains.

In conclusion, our results support the growing scientific evidence that sialylation of LOS could be advantageous for the fitness and infectivity potential of *C. jejuni* in different reservoirs and hosts. The present study revealed a correlation between MLST clonal complexes and certain LOS locus classes. This correlation needs to be investigated further, possibly to determine if it underlies a biological advantage for *C. jejuni* in colonizing birds and surviving in the environment.

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EXPERIMENTAL METHODS

Isolate collection and growth conditions. All chicken meat (n = 76) and human (n = 40) isolates were identified as *C. jejuni* by using multiplex PCR as described by Vandamme *et al*. [41]. The food-related bacterial collection consisted of 76 *C. jejuni* isolates from 74 chicken meat preparation samples. The term "chicken meat preparation" refers to portioned, cut, or minced meat to which other ingredients (e.g., salt, spices, seasoning mix, marinade, or sauce) may have been added, though the cut surface retains the characteristics of raw meat [42]. The chicken meat samples were from five Belgian companies and were collected in a survey between February and November 2007 [21]. In addition, 40 clinical *C. jejuni* isolates were also investigated. Strains in the human collection were isolated from the stool specimens of 39 patients admitted with sporadic cases of diarrhea and were provided by the same Belgian hospital laboratory in Brussels. Human isolates were cultured over the period from May to September 2007, and related clinical data were supplied. Supplementary Results, Data File 1 provides details on the origins and sampling dates for all isolates, in addition to data on the sources and processing batches of chicken meat samples.

The isolate collection was stored at –80°C in sterile full horse blood (E & O Laboratories, Bonnybridge, United Kingdom) and had been minimally subcultured before storage and subsequent testing. When required, isolates were cultured from the frozen stock for 24 h on blood agar plates (Muller-Hinton agar base CM337 [Oxoid, Basingstoke, United Kingdom] supplemented with 5% [vol/vol] full horse blood [E & O Laboratories]) under a microaerobic atmosphere at 37°C.

PCR screening of LOS locus classes. DNA was prepared by alkaline lysis as described previously (10). Primer sets specific for LOS locus classes A/B, B, C, D, and E were used [9]. LOS class A isolates were distinguished from class B isolates as those amplified by PCR primer A/B but not after successive screening by a class B-specific primer. PCR assays were performed using an iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA) with a touchdown program consisting of an initial denaturation step of 5 min at 94°C; 10 cycles of 1 min at 94°C, 1 min at 60°C (with the temperature initially reduced by 1°C per cycle until it reached 50°C), and 1 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. PCRs were performed in a 25-µl volume; each reaction mixture contained 2 µl of DNA template and a PCR mixture consisting of 1x buffer, 200 µM each deoxynucleoside triphosphate, 50 pmol of each PCR primer, 3 mM MgCl2, and 2 U of Taq polymerase per reaction (final concentrations). All PCR reagents were from Invitrogen, Merelbeke, Belgium. DNAs from GBS/MFS- and enteritis-associated *C. jejuni* strains for which the LOS loci had been identified previously [15] were used as PCR-positive controls.

MLST. All *C. jejuni* isolates were characterized by MLST on the basis of primers for seven gene targets for each isolate (*aspA* [encoding aspartase A], *glnA* [glutamine synthase], *gltA* [citrate synthase], *glyA* [serine hydroxymethyltransferase], *pgm* [phosphoglucomutase], *tkt*

[transketolase], and *uncA* [ATP synthase alpha subunit]) under conditions described previously [24, 43]. All allelic sequences were queried against the *C. jejuni* MLST database (http://pubmlst.org/*Campylobacter*/), developed by Keith Jolley and Man-Suen Chan and sited at the University of Oxford. Alleles already present in the database were assigned the numbers given there; novel alleles and sequence types (STs) were submitted to the *C. jejuni* MLST database and assigned new numbers.

PFGE. PFGE was performed using SmaI-digested fragments of bacterial chromosomal DNA as previously described [44]. Gel patterns were analyzed using GelCompar software (Applied Maths, Kortrijk, Belgium) with the band tolerance set at 1.5% [45].

Invasion assay. A panel of 52 *C. jejuni* isolates from chicken meat (n = 30) and from human enteritis cases (n = 22) was selected for the invasion assay. The 52 isolates were randomly selected in relation to their PCR-assigned LOS locus classes, as follows: 12 isolates each of classes B, C, and E, 11 isolates of class D, and all 5 isolates identified as class A. The gentamicin protection assay used in this study was the same as that described by Louwen *et al*. (2008) [15]. Briefly, Caco-2 cells were seeded and grown to confluence (5.0 x 10^6 cells) in 6-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Monolayers were incubated with *C. jejuni* at a multiplicity of infection of 100:1 for 4 h at 37°C under a 5% CO_2 atmosphere. Cells were then washed with prewarmed Dulbecco's modified Eagle's medium (Invitrogen, Breda, The Netherlands) and incubated for another 2 h in 2 ml Dulbecco's modified Eagle's medium containing gentamicin (480 µg ml–1) to kill extracellular bacteria. After the gentamicin kill period, the infected monolayers were washed three times with Hanks' buffered salt solution (Invitrogen, Breda, The Netherlands) and lysed with 0.1% Triton X-100 (Cornell, Philadelphia, PA) in phosphate-buffered saline (Invitrogen, Breda, The Netherlands) for 15 min at room temperature to release the intracellular bacteria. The number of viable bacteria released from the cells was assessed after serial 10-fold dilutions of the lysates on blood agar plates (Becton Dickinson, Breda, The Netherlands). Percentages of internalization were calculated, based on four tests per strain, by performing two independent assays, each done in duplicate on separate occasions and by different technicians working in parallel. A Penner serotype *C. jejuni* reference strain (P4; low invasiveness [15]) was used as an internal control strain to account for interexperimental variation.

PCR screening of virulence-related genes. The 52 *C. jejuni* isolates selected for the invasion assay were also screened for the presence of certain adhesion-, invasion-, and toxin-related genes. Previously published PCR primers and conditions were used for the detection of *ceuE* [46], *cadF* [47], *ciaB* [48], *pldA* [48], *cdtA* [38], *cdtB* [48], and *cdtC* [48].

Statistical analysis. Correlation between categorical independent variables (isolates' origins, LOS classes, and MLST clonal complexes) and the invasion phenotype (the dependent variable) was tested. The dependent variable in this analysis is estimated as the number of *C. jejuni* CFU on blood agar plates after plating from Caco-2 monolayer lysates. The CFU count on agar plates follows a Poisson distribution, the distribution used to fit counts (0) of events

that should be randomly distributed in space and time. Thus, the analysis was conducted using generalized linear models, starting with Poisson regression analysis. In case of extra-Poisson variation, negative binomial regression was used to account for the overdispersion in the data. Differences in the number of *C. jejuni* isolates between comparison groups (e.g., differences in numbers of isolates per clonal complex) were accounted for by applying frequency-weighting procedures. Model analysis was performed and fitted in Stata statistical software, version 8.0. [49].

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Supplemental Table 1

LOS class [a]	Origin [a]	Isolate [a,b]	Isolation date [a]	Sequence type [c]	Clonal complex [d]	Meat sample no [e]
A (n = 5)	Chicken (n = 4)	272-2	3 May 2007	257	257	M-23
		187-1	11 April 2007	3550*	21	F-4
		157-1	3 April 2007	704	NA	P-8
		501-3	21 August 2007	883	21	F-11
	Human (n = 1)	R357	8 September 2007	262	21	
B (n = 26)	Chicken (n = 14)	442-1	29 June 2007	122	206	M-32
		272-1	3 May 2007	257	257	M22
		353-2	6 June 2007	305	574	M-24
		490-1	14 Augst 2007	305	574	M-V
		419-1	22 June 2007	3548*	NA	M-28
		422-2	22 June 2007	3548*	NA	M-30
		344-1	1 June 2007	42	42	P-13
		353-1	6 June 2007	464	NA	M-24
		538	10 September 2007	48	48	P-17
		307-1	22 May 2007	572	206	C-8
		285-1	8 May 2007	572	206	N-6
		134-1	27 March 2007	572	206	C-4
		40-2	27 February 2007	572	206	C-1
		495-3	14 August 2007	572	206	C-12
	Human (n = 12)	D801	10 July 2007	1377	42	
		E541	10 August 2007	42	42	
		E540	6 August 2007	1724	460	
		G373	31 July 2007	572	206	
		H397	8 August 2007	572	206	

Supplemental Table 1 (continued)

LOS class [a]	Origin [a]	Isolate [a, b]	Isolation date [a]	Sequence type [c]	Clonal complex [d]	Meat sample no [e]
		J152	28 August	122	206	
		K328	19 September 2007	3552*	446	
		L406	9 July 2007	1377	42	
		L407	9 july 2007	3553*	NA	
		O197	26 September 2007	607	607	
		P243	27 July 2007	3554*	NA	
		Z145	1 May 2007	450	446	
C (n =30)	Chicken (n = 22)	204-1	12 April 2007	19	21	M-18
		530-1	3 September 2007	19	21	F-12
		201-3	12 April 2007	2037	NA	M-16
		39-2	27 February 2007	2324	NA	F-2
		271-1	3 May 2007	25	45	M-22
		240	24 April 2007	3128	21	C-6
		654	20 November 2007	3544*	NA	M-45
		214-3	17 April 2007	3546*	NA	N-5
		429-2	26 June 2007	44	21	C-11
		109-1	20 March 2007	50	21	N-2
		559	18 September 2007	50	21	M-36
		629	6 November 2007	50	21	C-V
		79E1	13 March 2007	50	21	F-3
		135-1	27 March 2007	50	21	C-5
		202-1	12 April 2007	50	21	M-17
		221-1	18 April 2007	50	21	P-10
		581	9 October 2007	50	21	F-10
		499-3	21 August 2007	50	21	F-Z
		51-1	6 March 2007	50	21	P-4
		75-1	13 March 2007	50	21	F-3
		408	19 June 2007	53	21	N-7
		357-1	6 June 2007	990	257	M-26
	Human (n=8)	A842	4 June 2007	21		
		C463	21 August 2007	21		
		D807	20 September 2007	21		
		G374	30 September 2007	21		
		L408	27 July 2007	21		
		M766C	8 June 2007	21		
		P242	10 July 2007	21		
		V485	6 May 2007	21		
D (n = 21)	Chicken (n = 15)	136-1	27 March 2007	2258	NA	C-5
		2-2	7 February 2007	257	257	F-1
		46-4	1 March 2007	257	257	M-1
		48-1	1 March 2007	257	257	M-3
		158-1	3 April 2007	2883	NA	P-8
		478	10 July 2007	2899	446	P16
		355-1	6 June 2007	354	354	M-25
		356-1	6 June 2007	354	354	M-26

Supplemental Table 1 (continued)

LOS class [a]	Origin [a]	Isolate [a, b]	Isolation date [a]	Sequence type [c]	Clonal complex [d]	Meat sample no [e]
		603-1	23 October 2007	354	354	M-38
		239-1	24 April 2007	354	354	F-6
		537	10 September	45	45	P-17
		498-1	14 August 2007	45	45	C-12
		8-2	15 February 2007	775	52	P-2
		256-1	27 April 2007	990	257	M-19
		199-1	12 April 2007	990	257	M-14
	Human (n = 6)	A841	31 May 2007	354	354	
		C457	11 June 2007	354	354	
		C458	21 June 2007	354	354	
		E539	27 July 2007	969	354	
		T317	13 June 2007	2398	NA	
		V488	12 June 2007	775	52	
E (n = 19)	Chicken (n = 9)	501-1	21 August 2007	2219	45	F-11
		556-2	18 September 2007	2807	443	M-33
		208-1	17 April 2007	443	443	M-19
		268-2	3 May 2007	45	45	M-21
		358	6 June 2007	45	45	M-27
		373E1	8 June 2007	45	45	N-6
		122E	22 March 2007	45	45	P-7
		41-2	27 February 2007	45	45	C-2
		146	28 March 2007	51	443	M-10
	Human (n = 10)	B1093	3 July 2007	583	45	
		B1096	10 September 2007	583	45	
		Z146	22 August 2007	400	353	
		C461	13 July 2007	400	353	
		D803	30 July 2007	137	45	
		D804	30 July 2007	137	45	
		D805	30 July 2007	137	45	
		M768	7 July 2007	443	443	
		R356	24 July 2007	267	283	
		S478	11 September 2007	443	443	
Others (n = 15)	Chicken (n = 12)	393-2	15 June 2007	1759	NA	C-10
		423	22 June 2007	2037	NA	M-31
		6-2	15 February 2007	2037	NA	P-2
		238-2	24 April 2007	2324	NA	F-5
		149	28 March 2007	257	257	M-12
		1-2	7 February 2007	257	257	F-1
		7-2	15 February 2007	257	257	P-2
		560	18 September 2007	3544*	NA	M-37
		652	20 November 2007	3544*	NA	M-43
		121-1	22 March 2007	3544*	NA	P-7
		222-1	18 April 2007	50	21	P-10
		461	3 July 2007	905	NA	N-8

Supplemental Table 1 (continued)

LOS class [a]	Origin [a]	Isolate [a, b]	Isolation date [a]	Sequence type [c]	Clonal complex [d]	Meat sample no [e]
		B1094	2 August 2007	48	48	
		C456	7 September 2007	354	354	
		L404	13 June 2007	3546*	NA	

Supplemental Table 1 Correlation between genotypic diversity, lipo-oligosaccharide gene locus class variation. [a] Isolates from humans (n = 40) and chicken meat (n = 76) are grouped according to their LOS classes, isolation dates, and MLST results. [b] Isolates 272-2 and 272-1 (classes A and B, respectively) are from the same chicken meat sample. Isolates D803 and D804 (class E) are from the same human sample. [c] Asterisks indicate novel sequence types, first reported in this collection. [d] NA, not assigned to a defined clonal complex (MLST online database last accessed in September 2008). [e] Given as the code for the company of origin-number of the processing batch.

Supplemental Table 2

	No. of isolates (frequency [%]) from:	
MLST clonal complex	Chicken meat (n = 76)	Human diarrhea specimens (n = 40)
Not assigned	18 (23.7)	4 (10.0)
CC-21	19 (25.0)	9 (22.5)
CC-42	1 (1.3)	3 (7.5)
CC-45	9 (11.8)	5 (12.5)
CC-48	1 (1.3)	1 (2.5)
CC-52	1 (1.3)	1 (2.5)
CC-206	6 (7.9)	3 (7.5)
CC-257	11 (14.5)	
CC-283		1 (2.5)
CC-353		2 (5.0)
CC-354	4 (5.26)	5 (12.5)
CC-443	3 (3.95)	2 (5.0)
CC-446	1 (1.3)	2 (5.0)
CC-460		1 (2.5)
CC-574	2 (2.63)	
CC-607		1 (2.5)

Supplemental Table 2 Comparison of frequency distribution of MLST clonal complexes in *C. jejuni* isolates from Belgian chicken meat preparations and human diarrhoeal samples.

Supplemental Table 3

Isolate no.	Strain	Los class	Origin	Sequence type	Clonal complex	% of inoculum internalized (avg ± SD)
1	442-1	B	Chicken	ST-122	CC-206	0.2614 ± 0.072
2	344	B	Chicken	DT-42	CC-42	0.2476 ± 0.008
3	353-1	B	Chicken	ST-464	NA[c]	0.2060 ± 0.061
4	H397	B	Human	ST-572	CC-574	0.2031 ± 0.053
5	408	C	Chicken	ST-53	CC-21	0.1811 ± 0.023
6	499-3	C	Chicken	ST-50	CC-21	0.1686 ± 0.034
7	D803	E	Human	ST-137	CC-45	0.1395 ± 0.021
8	G374	C	Human	ST-21	CC-21	0.1293 ± 0.029
9	135-1	C	Chicken	ST-50	CC-21	0.1277 ± 0.014

10	79-E1	C	Chicken	ST-50	CC-21	0.1267 ± 0.022
11	501-3	A	Chicken	ST-883	CC-21	0.1068 ± 0.050
12	490-1	B	Chicken	ST-305	CC-206	0.1051 ± 0.015
13	285-1	B	Chicken	ST-572	CC-206	0.0880 ± 0.025
14	L408	C	Human	ST-21	CC-21	0.0818 ± 0.028
15	187-1	A	Chicken	ST-3550[b]	CC-21	0.0791 ± 0.012
16	C457	D	Human	ST-354	CC-354	0.0790 ± 0.006
17	495-3	B	Chicken	ST-372	CC-206	0.0639 ± 0.009
18	C458	D	Human	ST-354	CC-354	0.0624 ± 0.032
19	J152	B	Human	ST-122	CC-206	0.0544 ± 0.023
20	G373	B	Human	ST-572	NA[c]	0.0539 ± 0.005
21	D807	C	Human	ST-53	CC-21	0.0477 ± 0.003
22	P242	C	Human	ST-1728	CC-21	0.0463 ± 0.018
23	L406	B	Human	ST-1377	CC-42	0.0352 ± 0.002
24	R357	A	Human	ST-262	CC-21	0.0331 ± 0.007
25	R356	E	Human	ST-267	CC-283	0.0306 ± 0.016
26	307-1	B	Chicken	ST-572	CC-206	0.0300 ± 0.012
27	S478	E	Human	ST443	CC-443	0.0300 ± 0.001
28	501-1	E	Chicken	ST-2219	CC-45	0.0288 ± 0.017
29	358-1	E	Chicken	ST-45	CC-45	0.0258 ± 0.009
30	208	E	Chicken	ST-443	CC-443	0.0224 ± 0.001
31	239-1	D	Chicken	ST-354	CC-354	0.0184 ± 0.002
32	272-2	A	Chicken	ST-257	CC-257	0.0175 ± 0.006
33	2	D	Chicken	ST-257	CC-257	0.0170 ± 0.001
34	530	C	Chicken	ST-19	CC-21	0.0156 ± 0.001
35	V488	D	Human	ST-775	CC-52	0.0143 ± 0.002
36	373	E	Chicken	ST-45	CC-45	0.0124 ± 0.001
37	M768	E	Human	ST-443	CC-443	0.0115 ± 0.002
38	8	D	Chicken	ST-775	CC-52	0.0106 ± 0.001
39	A841	D	Human	ST-354	CC-354	0.0101 ± 0.004
40	46	D	Chicken	ST-257	CC-257	0.0097 ± 0.001
41	157-1	A	Chicken	ST-704	NA	0.0088 ± 0.004
42	E541	B	Human	ST-42	CC-42	0.0065 ± 0.002
43	355-1	D	Chicken	ST-354	CC-354	0.0062 ± 0.004
44	D805	E	Human	ST-137	CC-45	0.0052 ± 0.001
45	603	D	Chicken	ST-354	CC-354	0.0052 ± 0.001
46	C463	C	Human	ST-19	CC-21	0.0051 ± 0.001
47	199	D	Chicken	ST-990	CC-257	0.0030 ± 0.001
48	B1096	E	Human	ST-583	CC-45	0.0024 ± 0.001
49	559-3	C	Chicken	ST-50	CC-21	0.0019 ± 0.001
50	146	E	Chicken	ST-51	CC-443	0.0010 ± 0.002
51	M766C	C	Human	ST-50	CC-21	0.0002 ± 0.002
52	41-2	E	Chicken	ST-45	CC-45	0.0000

Supplemental Table 3 Invasion phenotyes of 52 *C. jejuni* strains assayed in Caco-2 cells in relation to their LOS locus classes and MLST genotypes [a]. [a] Results are given in descending order according to the percentage of the starting viable inoculum internalized into cells after gentamicin resistance assays. The space after isolate 14 marks the cutoff of 0.1% of the inoculum internalized (the standard deviation is considered for isolates 13 and 14). [b] Novel sequence type. [c] NA, not assigned to a defined clonal complex (MLST online database last accessed in September 2008).

Chapter 6

Lack of association between the presence of pVir plasmid and bloody diarrhea in *Campylobacter jejuni* enteritis

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ABSTRACT

The main mechanisms by which *Campylobacter jejuni* causes diarrhea are unknown. In contrast to a recent communication, we here report the absence of an association with plasmid pVir in patients with *C. jejuni*, who developed bloody diarrhea in The Netherlands and suggest a role for other virulence determinants.

Campylobacter jejuni is the leading cause of human bacterial gastroenteritis worldwide. A great variety of clinical symptoms is observed in patients infected with *C. jejuni* ranging from asymptomatic carriership and mild watery diarrhea, to severe and bloody diarrhea with fever. Disease complications include bacteremia, reactive arthritis and acute post-infectious neuropathies: the Guillain-Barré (GBS) and Miller Fisher (MFS) syndromes.

The virulence factors involved in the pathogenesis of *C. jejuni* diarrhea are still poorly characterized. Various mechanisms have been reported to be involved including adherence, cellular invasion, and toxin production. Motility and multiple adhesins appear to play a role in intestinal adherence and colonization [1-2]. In addition, lipo-oligosaccharide (LOS) structures have been found to be involved in the pathogenesis of post-infectious neuropathy by molecular mimicry with human gangliosides [3]. A significant proportion of *C. jejuni* harbors plasmids and the contribution of plasmids in the pathogenesis and antimicrobial resistance of *Campylobacter* infections have been studied since the early eighties [4]. The plasmid pVir has been implicated in the virulence of *C. jejuni* [5]. pVir contains genes for homologues of Com and Vir proteins, that are presumably involved in DNA uptake or protein transport via a putative bacterial type IV secretion machinery [5]. More recently, Tracz *et al* [6] identified pVir in 17 out of 104 (17%) clinical *C. jejuni* isolates and found that isolates containing pVir were associated with the presence of a tetracycline-resistance plasmid. In addition, they report a significant association of the presence of pVir with bloody diarrhea, and suggest an important role of pVir in the pathogenesis of more severe invasive *Campylobacter* infections.

We detected pVir and the Tet(O) gene in *C. jejuni* strains, isolated from 125 well characterized community-based Dutch patients and analyzed the association with bloody diarrhea [7]. In addition, as preliminary studies in our laboratory (data not shown) indicated that GBS/MFS-related *C. jejuni* are more invasive in vitro, we wondered whether the presence of pVir is elevated among a particular set of 21 *C. jejuni* strains isolated from GBS/MFS patients in The Netherlands.

Plasmid and chromosomal DNA was isolated using QIAamp DNA Mini Kit (Qiagen). pVir and Tet(O) were detected by PCR using the protocol described by Tracz *et al* [6]. *C. jejuni* 81176 was used as a positive control. Tetracycline MIC's were determined using the E-test method on Mueller Hinton agar supplemented with 5% sheep blood. *C. jejuni* ATCC 33560 was used as quality control strain.

pVir was detected in 4/125 (3%) enteritis strains, in 1/17 (6%) of the GBS-related strains and was not detected in the four MFS-related strains. Tet(O) was found in 41/125 (32%) enteritis strains, in 4/17 (24%) of the GBS related strains, and in 1 out of 4 MFS-related strains (25%). All five GBS/MFS-related strains that contained Tet(O) had MIC's for tetracycline of > 256 mg/L. The strains that did not harbor the Tet(O) gene had MICs for tetracycline that ranged from 0.064 – 0.5 mg/L. The prevalence of Tet(O) among pVir negative strains was 33%. Bloody diarrhea was reported in 48 out of the 125 patients. Only one of the 48 patients was infected by a pVir positive *C. jejuni* strain.

Our data point towards a remarkably low prevalence of pVir in *Campylobacter* strains in the Netherlands in contrast to a recent report from Canada [6]. Furthermore, no significant difference was observed in the prevalence of pVir in 125 enteritis strains compared to Dutch GBS and MFS strains, although the number of GBS/MFS strains was small as these isolates are infrequently isolated. The absence of an association between the presence of pVir and bloody stools suggests that other virulence factors may be involved in the development of bloody diarrhea. A suggestion in this direction was recently made by Champion *et al*., who proposed a serine protease, encoded by *Cj*1365 as a virulence factor involved in the development of bloody diarrhea [8]. Previously, serine proteases have been described in Enterobacteriaceae and are thought to be involved in the development of bloody diarrhea caused by *Escherichia coli* [9].

One must emphasize that the loss of plasmids during subculture cannot be excluded, although the observation on the stability of pVir and the tetracycline resistance plasmid in strain 81176 do suggest that this appears to be an infrequent event [5]. Alternatively, comparing the data of Tracz *et al*. and ours, one wonders whether patient and geographical characteristics may explain the observed differences. Our patient group comprised of 130 well characterized, community based Dutch patients, none of which requiring hospitalization during the course of the disease. Differences in disease severity or geographical locale may eventually parallel the great variety in prevalence of pVir in *Campylobacter*. Thus, our data add to those of Tracz *et al*. and further strengthen their conclusions that more studies are needed to assess the association of pVir and other virulence markers among the widening spectrum of *C. jejuni* that cause disease in humans.

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Chapter 7

Can *Campylobacter coli* induce Guillain-Barré Syndrome?

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ABSTRACT

There is an increase in evidence that not only *C. jejuni*, but also other *Campylobacter* species precede the post-infectious Guillain-Barré Syndrome (GBS). We here report a detailed analysis on two *Campylobacter coli* strains, both isolated from a GBS patient. Remarkably, we were unable to provide any evidence that molecular mimicry plays a role in the pathogenesis of GBS that is preceded by a *C. coli* infection.

Campylobacter jejuni enteritis is the most frequently identified infection preceding the Guillain-Barré Syndrome (GBS) and neural damage is thought to be induced through molecular mimicry between *C. jejuni* lipo-oligosaccharide (LOS) and human gangliosides [1]. It has been questioned whether or not other *Campylobacter* species, including *C. curvus*, *C. upsaliensis* and *C. coli*, could be similarly involved [2-4]. This is relevant because it would imply that bacterial factors considered important in the etiology of GBS crossed species barriers. Two prior reports have appeared where *C. coli* putatively were associated with a case of GBS [3-4].

We present two female patients with GBS, one from the Netherlands (patient GB50) and one from France (patient 664H2004). From a faecal specimen obtained for both patients, a *C. coli* strain was isolated. On the basis of surface protein profiling, the strains were unequivocally demonstrated to belong to the species *C. coli* (results not shown). The strains were encoded GB50 and 664H2004, respectively, and stored at −80°C. For patient GB50, a serum sample obtained at the acute GBS phase was available. This sample was also stored at −80°C.

Strains were grown on Mueller-Hinton agar at 37°C for 48 h, after which DNA was extracted, as described by Pitcher *et al*. [5]. Amplified fragment length polymorphism (AFLP) analysis was performed, as described by Duim *et al*. [6]. In brief, 1 µg of genomic DNA was digested with the HindIII-HhaI restriction enzyme combination and site-specific adaptors were ligated to the restriction fragments. Primers complementary to the adaptor and restriction site sequence were used in pre-selective and selective polymerase chain reaction (PCR) amplifications. The amplified and fluorescently labeled fragments were loaded on an ABI Prism 377 automated sequencer. GeneScan version 3.1 (Applied Biosystems) was used for data collection, and the AFLP profiles were imported, using the CrvConv filter, in BioNumerics 4.61 (Applied Maths, Belgium) for normalization and further analysis.

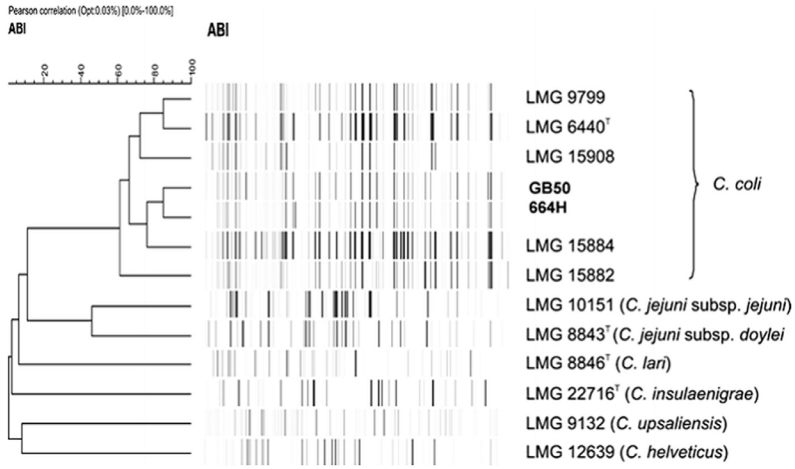

Figure 1 Amplified fragment length polymorphism (AFLP) analysis of the two *Campylobacter coli* strains isolated from patients GB50 and 664H2004. Note that, despite their diverse geographic origins, these strains cluster, but clearly fall within the C. *coli* cluster.

The obtained AFLP profiles were included in an in-house AFLP reference frame, containing profiles from all known *Campylobacter* species. Similarity between the normalized fingerprints was determined by the Pearson product moment correlation coefficient and a UPGMA dendrogram was constructed. The profiles from both isolates clearly formed a cluster together with *C. coli* reference strains (**Fig. 1**).

Using LOS gene cluster-specific PCR tests, the 664H2004 strain was demonstrated to harbor a B-type gene cluster [7]. For GB50, neither the *cst-II* nor the *cgtA* gene was shown to be present. This convincingly demonstrates that this strain does not harbor the neuropathy-associated LOS gene cluster types A, B or C. Using primers based on the *C. coli* RM2228 LOS gene cluster, we amplified the LOS locus of GB50, but we could not amplify the LOS locus of 664H2004. Complete sequencing of the novel GB50 LOS gene cluster revealed 12 open reading frames (ORFs) that included five putative glycosyltransferases and a few ORFs seemingly unrelated to LOS biosynthesis (**Table 1**).

Source sequence		1…11261	
Nucleotide position	**Gene orientation**	**Gene annotation**	**Putative gene function**
1 – 108	+	waaC	Heptosyltransferase I
101 – 988	+	htrB	Lipid A biosynthesis acyltransferase
985 – 2538	+	Orf3	Putative glycosyltransferase
2535 – 3587	+	Orf4	Putative glycosyltransferase
3584 – 4783	_	Orf5	Putative glycosyltransferase
4911 – 6215	+	Orf6	Putative CDP-glycerol:polyglycerophosphatase
6212 – 7246	+	Orf7	Hypothetical protein
7316 – 8335	+	Orf8	Transposase-like insertion element
8214 – 8924	+	Orf9	Hypothetical protein
8917 – 9837	+	Orf10	Putative DNA methyltransferase
10021 – 10437	+	Orf11	Putative glycerol-3-phosphate cytidyltransferase
10456 – 11261	-	waaV	Putative glycosyltransferase

Table 1 Information on the lipo-oligosaccharide (LOS) gene cluster for strain GB50 (GenBank accession number EU374214)

The LOS of *C. coli* strain GB50 did not bind cholera toxin, hence, the presence of a GM1-like ganglioside mimic could be excluded. The acute phase pre-treatment serum from patient GB50 showed a high level of IgG activity to the LOS from the GB50 strain (**Table 2**). This activity was significantly higher than in the serum from ten healthy blood donors. In addition, IgM activity was found for this LOS in the serum taken from patient GB50, although it was less than the IgG activity. Some of the healthy blood donors also showed this elevated level of IgM. Probing the LOS with six specific monoclonal anti-ganglioside antibodies (DG-1, DG-2, TBG-3, EG-7, EG-3 and EG-1) did not reveal any reactivity.

	GB50	**664H2004**
Genotyping strains		
LOS biosyntheis cluster	Non-typable (no sialic acid incorporating enzymes encoded	Class B (does contain Cst-II
Structure of LOS (MS/MS analysis)		
Presence sialic acids	-	Di-NeuAc
Binding studies with LOS		
Cholera toxin B-subunit	-	-
Anti-ganglioside mAbs[1]	-	-
IgG in serum patient GB50	++[2]	-
IgM in serum patient GB50	+[2]	-
Serum anti-ganglioside antibodies[3]		
IgG	GM1 (6400), GD1b (400)	n.a.
IgM	GM1 (800), GD1b (200)	n.a.
IgA	GM1 (200), GD1b (200)	n.a.
Cross-reactivity serum antibodies Anti-GM1 IgG in serum GB50[4]	-	-

Table 2 Characterisation of *Campylobacter coli* strains isolated from two patients with Guillain-Barré Syndrome (GBS). n.a.; not available; [1] Monoclonal antibodies DG-1 (to GM1), DG-2 (to GM1/GD1b/GA1), TBG-3 (to GD1a), EG-7 (to GD1b), EG-3 (to GQ1b) and EG-1 (to GT1a/GQ1b); [2] Significantly higher than antibody activity to this LOS in serum from ten healthy blood donors; [3] Serum IgG, IgM and IgA tested to GM1, GM2, GD1a, GD1b, GD3 and GQ1b; [4] The cross-reactivity of 1:400 diluted serum anti-GM1 IgG antibodies from patient GB50 to LOS from *C. coli* GB50 and 664H2004 were determined by inhibition enzyme-linked immunosorbent assay (ELISA)

These monoclonal antibodies were raised by immunisation with *C. jejuni* LOS and bound to various (combinations of) gangliosides (**Table 2**). Interestingly, regular mass spectrometry analysis on O-deacylated *C. coli* LOS [1] did not reveal a structural overlap between this LOS and the previously determined *C. jejuni* LOS structures. It appeared that the GB50 LOS did not contain sialic acid based on MS/MS analysis. The LOS from the 664H2004 strain was shown to contain di-NeuAc based on MS/MS analysis.

The serum from patient GB50 was tested in a standardised enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to the gangliosides GM1, GM2, GD1a, GD1b, GD3 and GQ1b [8]. The serum was positive for IgG, IgM and IgA antibodies to the gangliosides GM1 and, to a lesser extent, to GD1b (**Table 2**). These gangliosides share the terminal Gal (β1–3) GalNac to which these antibodies are probably directed. Accordingly, the serum from this patient contained high IgG, IgM and IgA activity to the non-ganglioside gly*coli*pid asialo-GM1, which has the same terminal disaccharide. Such a structure, however, was not identified in the LOS of the *C. coli* isolate from this patient. This suggests that this patient might have been co-infected with another organism expressing a GM1 mimic, possibly a *C. jejuni* strain, responsible for a non-GBS-related induction of a cross-reactive antibody response to GM1. In inhibition ELISA experiments, anti-GM1 IgG activity in 1:400 dilutions of serum from GB50 was not reduced by LOS from this strain in concentrations of up to 200 µg/ml, demonstrating that these antibodies do not cross-react with the LOS from the *C. coli* isolate from the patient. In control studies, this antibody activity was reduced to a level of less than 5% by pre-incubation with LOS from a *C. jejuni* isolate GB2 from another GBS patient, in which mimicry with GM1 was previously demonstrated by MS [9].

Overall, we have comprehensively characterized two *C. coli* strains isolated from GBS patients with a variety of state of the art technologies. One strain (664H2004) harbored disialylated LOS, but, unfortunately, no serum was available to measure either anti-ganglioside or anti-LOS antibodies in the GBS patient herself. The other strain from the Dutch patient (GB50) had non-sialylated LOS, which showed no binding to cholera toxin or to monoclonals specific for several gangliosides. However, the serum from patient GB50 from which *C. jejuni* GB50 was isolated did contain antibodies to the LOS from this strain, indicating that this infection led to a specific immune response to *C. coli* strain GB50. The role of this immune response in the pathogenesis of GBS is currently unknown, although other examples of GBS patients infected with non-ganglioside mimicking strains of *C. jejuni* have been described [10]. The serum from GB50, however, also contained antibodies to GM1 and GD1a, which do not cross-react with LOS from GB50, as expected, although they do cross-react with LOS from a *C.*

jejuni with a known GM1 mimic. These serological findings in patient GB50 strongly suggest that this patient was infected with at least two micro-organisms, one of which induced the anti-ganglioside antibodies leading to GBS (possibly a *C. jejuni* strain), and *C. coli*, which also induced an immune response, but probably did not trigger GBS.

In summary, we could not produce any evidence that molecular mimicry plays a role in the pathogenesis of GBS that is preceded by a *C. coli* infection. It is still not clear whether *C. coli* isolated from the faeces of GBS patients can play a role in the development of GBS. Additional GBS patients and their *C. coli* strains need to be reported and characterized in detail in order to better understand the pathogenesis of GBS in these patients.

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Chapter 8

Discussion, conclusions and remaining research questions

DISCUSSION, CONCLUSIONS AND REMAINING RESEARCH QUESTIONS

Sialic acid containing sugar structures play an important role in eukaryotic and prokaryotic biology [1]. In eukaryotes sialic acid sugar structures are used to distinguish self from non-self [1]. Microorganisms explore these structures for infection [2], for example bacteria and viruses secret sialidases to unmask binding receptors protected by sialic acid sugar structures [3,4]. Other bacteria have generated toxins that use sialic acid sugar structures for binding, enabling cell entry to disorganize intra-cellular eukaryotic cells in advantage to the pathogen [5].

A subset of *C. jejuni* bacteria is able to express sialic acid containing sugar structures on their cell envelope (sialylated LOS) in such a way that this gives molecular mimicry with eukaryotic gangliosides [6]. For *C. jejuni* and other bacterial species it has been established that sialylation of cell envelope structures, helps to evade host immune defenses in man [7-11]. Contradicting reports were published on the role of such *C. jejuni* ganglioside mimics in cellular invasion [6,10]. Another feature of *C. jejuni* ganglioside mimics, namely viral defense, was not explored yet. Therefore, an earlier report on LOS phase variation leading to increased bacteriophage resistance in *Haemophilus influenzae* [12] was of interest. Next to serum resistance and host invasion, expression of a sialylated cell envelope by *C. jejuni* and other bacterial species has been repetitively shown to correlate with human disease complications [6,8,13-17]. Unfortunately, the pathogenic mechanism(s) behind this process remains to be elucidated. In this thesis we generated new insights on why a subset of *C. jejuni* isolates express ganglioside mimic structures.

In **Chapter 2** we revealed that ganglioside mimics are important in bacteriophage defense and that LOS modification with sialic acids also affects the canonical bacteriophage defense system CRISPR-Cas.

In **Chapter 3 – 5** we established that ganglioside mimics are needed for increased efficiency to bind to, invade in and translocate across intestinal epithelial cells, with a large number of chicken- and clinical-derived *C. jejuni* isolates. In **Chapter 6** we were not able to link the virulence plasmid pVir with bloody stools, whereas this was shown by others. In **Chapter 7** we analyzed *C. coli* strains isolated from GBS patients. We found that these isolates did not express ganglioside mimics. This could indicate that although bacterial ganglioside mimics are obviously associated with GBS, other factors could be involved in the induction of GBS. We will now discuss the chapters in more detail.

C. jejuni ganglioside mimics are an efficient bacteriophage defense system

In **Chapter 2** we show that ganglioside mimic expression is important for bacteriophage resistance in *C. jejuni*. These results were confirmed by knock-out mutagenesis and genetic complementation of the sialyltransferase gene *cst*-II, a previously established GBS marker [18]. The LOS biosynthesis genes belonging to the LOS loci class A or B are responsible for gan-

glioside mimic expression via sialyltransferase Cst-II and can be exchanged by HGT between *C. jejuni* isolates [19, 20]. As suggested by the putative function of the CRISPR-cas system [21-23], we addressed whether this system is involved as a selection factor in the uptake of such HGT exchangeable LOS loci. Our analysis of the CRISPR elements present in GBS- or enteritis-associated *C. jejuni* isolates did not reveal specific spacers in the CRISPR element that could be linked to the presence of the HGT exchangeable LOS biosynthesis genes. In contrast, the *cas* genes did harbor mutations that strongly associated with the presence of the HGT exchangeable LOS biosynthesis genes. The *cas* genes even harbored specific mutations that were found to be novel GBS markers, next to the established GBS marker *cst*-II [18]. Our results seem to support a mathematical model designed for CRISPR-Cas system dynamics by Bruce Levin [24]. His mathematical model suggested that the CRISPR-Cas system might became affected in a bacterial species [24] when cell envelope bacteriophage resistance is favoured above CRISPR-Cas resistance. Comparing *cst*-II positive with *cst*-II negative isolates enabled us to prove this mathematical prediction. We observed that *C. jejuni* isolates expressing *cst*-II mediated ganglioside mimics harbored a strongly reduced or even lacked a CRISPR-Cas system. Our bioinformatic analysis on other bacterial species (*N. meningitidis*, *H.* (*para*-) *influenzae* and *P. multocida*), all harboring sialyltransferases, corroborated the CRISPR-Cas *C. jejuni* observation. In these bacterial species presence of a sialyltransferase was also accompanied with a reduced or absent CRISPR-Cas system. Next, we were able to provide evidence using a Bayesian clock model [25], that the *cst*-II positive isolates were younger on an evolutionary time scale than the *cst*-II negative isolates. Indicative, that GBS-associated ganglioside mimic generated bacteriophage defense is younger in evolution then the CRISPR-Cas system.

Next to that, the CRISPR-Cas subtype to which *C. jejuni* belongs is mainly present in pathogenic vertebrate host-associated bacteria [22, 26]. The difference between this subtype and other CRISPR-Cas subtypes is not only its reduced size but also the presence of the *cas* gene *csn1* [22, 26]. Since *csn1* harbored specific synonymous and non-synonymous mutations that associated with disease outcome, we hypothesized involvement of *cas* gene *csn1* in *C. jejuni* pathogenesis. Indeed, our assays revealed that mainly the *cst*-II positive isolates were affected in virulence through knock out mutagenesis of the *csn1* gene. More strikingly was the observed difference in binding of GBS patient serum antibodies to the LOS of the *csn1* knock out in three GBS isolates, indicative for a biologically relevant change in membrane topology or composition.

C. jejuni ganglioside mimics are virulence factors.

Mucosal epithelial cells are the first to interact with enteric pathogens [27]. Previously, others showed for *N. meningitidis* and *H. influenzae* that presence of sialic acid containing sugar structures influenced the virulence potential of these bacterial species [8]. For *C. jejuni* this remained to be elucidated. In **Chapter 3 – 5** we show for *C. jejuni* that ganglioside mimics

are not only important as a bacteriophage defence system, but also contribute to *C. jejuni* virulence through increasing cellular binding, endocytosis and translocation across intestinal epithelial cells. Next, ganglioside mimic expressing isolates are associated with severe gastro-enteritis and bloody stools [13]. This led us to hypothesize that increased cellular endocytosis and translocation facilitated by ganglioside mimics forms a mechanistic basis for the clinical symptoms observed in *C. jejuni* diseased patients, which warrant further study. Sialylation of *C. jejuni* LOS structures by means of the classes A, B or C LOS loci, were also found to enhance cellular invasion [6,28,29], a result of increased binding to and endocytosis into Caco-2 intestinal epithelial cells (**Chapter 4**). We did find that no difference existed in invasion capacities between human or chicken obtained *C. jejuni* isolates [28].

For *C. jejuni* it is established that this bacterium can translocate across Caco-2 intestinal epithelial cells [30-33] and it is discussed that the mode of entry into intestinal epithelial cells determines the intracellular fate of *C. jejuni* [31, 34, 35]. Some intra-cellular bacteria have developed mechanisms to avoid delivery to lysosomes in intestinal epithelial cells to be able to survive, replicate, and translocate [36]. For *C. jejuni* it has been suggested that it survives intra-cellular by lysosomal escape [37]. So far, *C. jejuni* lysosomal escape has only been visualized in monkey kidney cells and Hela cells [37, 38]. Using Caco-2 intestinal epithelial cells, we did not find strong evidence for such lysosomal escape by *C. jejuni*.

Watson and Galan observed that intra-cellular *C. jejuni* bacteria required adapted culture methods for recovery [37]. Using these methods during our survival assay, we observed that both sialylated and non-sialylated *C. jejuni* isolates are efficiently killed in the Caco-2 cell. Only 1 - 3% of the *C. jejuni* bacteria survived 24 hours post-infection, which suggests that most of the *C. jejuni* bacteria were not able to escape the pathway to endo-lysosomal fusion and thereby killing.

Why would it be evolutionary advantageous for *C. jejuni* isolates to express ganglioside mimics if only 1 - 3% of sialylated and non-sialylated bacteria survives? By using fluorescent microscopy and endosomal protein markers, we showed that the presence of ganglioside mimic expression on *C. jejuni* is correlated with enhanced numbers of intra-cellular bacteria. Thus, if the same percentage of bacteria (sialylated versus non-sialylated) is killed per Caco-2 cell this will result in enhanced survival. Next, we demonstrate that the *C. jejuni* translocation process across Caco-2 intestinal epithelial cells is not very efficient, since only 1 - 3% of the invaded and surviving bacteria were finally able to translocate. We thus established that the differentiated Caco-2 intestinal epithelial cells are an efficient barrier to *C. jejuni* translocation, but that ganglioside mimic expression does facilitate the translocation process. Overall, our results thus suggest that ganglioside mimic expression is advantageous for the fitness and virulence potential of *C. jejuni* in different reservoirs and hosts. Ganglioside mimics thus belong to the repertoire of virulence factors that is used by a subset of *C. jejuni* bacteria to interact with Caco-2 intestinal epithelial cells and is probably useful for maintenance in different hosts.

A low prevalence of pVir and pTet in Dutch *C. jejuni* isolates.

In *C. jejuni* diseased patient's ganglioside mimic expression has been shown to be associated with severe gastro-enteritis and bloody stools [13]. In a study by Tracz *et. al.*, a virulence plasmid pVir was also suggested to be associated with increased virulence and bloody stools [39]. Analysis of 21 GBS/MFS- associated isolates and 125 matched controlled enteritis-associated isolates in **Chapter 6**, revealed that pVir was only present in 6% of the GBS and 3% of the enteritis-associated *C. jejuni* isolates [40]. Since we knew the clinical symptoms of the 125 matched enteritis control isolates enabled us to conclude that in The Netherlands pVir was not associated with severe gastro-enteritis or bloody stools. Another plasmid, pTet, thought to be associated with the presence of pVir was more prevalent in the Dutch *C. jejuni* isolates [40]. The pTet plasmid was detected in approximately 25% of the GBS/MFS isolates whereas in the enteritis isolates 32% harboured this plasmid [40]. The presence of pTet resulted in high resistance against tetracycline (> 256 µg/ml) [40]. The prevalence of (25 - 35%) tetracycline resistance is equal to that observed in Switzerland [41], whereas in other European countries, China and USA higher prevalences of tetracycline resistant *C. jejuni* isolates were documented [42-45]. The prevalence of pVir positive *C. jejuni* isolates in The Netherlands is thus unexpectedly low compared to an earlier study [39].

GBS INDUCTION AND *C. COLI* INFECTIONS.

We observed that *C. coli* infection can precede GBS, since from two different GBS patients, one form The Netherlands and one from France, a *C. coli* strain was isolated from the patient's stool at the time of hospitalization. *C. coli* isolation from GBS patients stands not on its own, since more researchers have isolated *C. coli* strains from GBS patients [46, 47]. Even though *C. coli* has been isolated from GBS patients it remains unsure if they are really the causative agent of GBS, since we and others have not found strong evidence yet that ganglioside mimicry plays an important role in *C. coli* associated GBS [46]. Co-infections have been suggested as a plausible explanation for such isolations [48], but these observations remain inconclusive. Not only *C. coli* but also *C. jejuni* induced GBS is thus far from being elucidated. Although the molecular mimicry hypothesis is a good candidate to explain *Campylobacter* induced GBS, this post-infectious complication still remains a box filled with questions.

Main conclusions

1. CRSIPR-Cas is involved in *C. jejuni* bacteriophage defense.
2. Ganglioside mimic expression is important for *C. jejuni* bacteriophage defense.
3. *C. jejuni* invasion into Caco-2 intestinal epithelial cells is enhanced by ganglioside mimic expression.
4. *C. jejuni* Caco-2 intestinal epithelial cell binding, endocytosis and epithelial translocation are facilitated by ganglioside mimic expression.
5. Ganglioside mimic expression is advantageous for *C. jejuni* fitness and virulence in different hosts.
6. pVir is not associated with bloody diarrhea in The Netherlands.
7. pTet associates with elevated tetracycline resistance in *C. jejuni* in The Netherlands.
8. *C. coli* is able to induce GBS, but more studies are needed to identify the factors involved.

REMAINING RESEARCH QUESTIONS

Sialylation of *C. jejuni* LOS is important in bacteriophage defense and virulence, but how sialylated LOS triggers GBS in susceptible patients still remains to be elucidated. In **Chapter 2,** for example, we found that knock out mutagenesis of the *cas* gene *csn1* resulted in increased GBS patient serum antibody binding to the sialylated LOS structures of 3 GBS *C. jejuni* isolates. In this **Chapter** we were not able to provide a clear answer on how this is possible. Unfortunately *csn1* complementation into these three GBS isolates was not successful, disabling us to attribute this phenomenon fully to Csn1 alone. Another remarkable observation with these three GBS *csn1* knock outs was that loss of *csn1* disabled the ability of *C. jejuni* to induce cell damage and translocation across an intestinal epithelial barrier. Supplementation of *csn1* into an isolate lacking a CRISPR-Cas system reduced its swarming behaviour, but increased its invasion potential, linking Csn1 thus indeed with virulence. Next, we did observe that Csn1 harbors a nuclear bipartite signal, which might be related to the loss of *C. jejuni* virulence through regulation of nuclear gene expression, which warrants further study. In **Chapter 2** two important questions remain to be answered; 1) How is the *C. jejuni cas* gene *csn1* involved in the characteristic GBS LOS immune response? 2) How is *C. jejuni* virulence regulated by Csn1?

Another feature that needs to be elucidated is the function of the *C. jejuni* CRISPR-Cas system in bacteriophage defense. Knock out mutagenesis of *csn1* did reveal involvement of this system in bacteriophage defense, but the processing of the CRISPR array and how bacteriophages are neutralized by this *C. jejuni* CRISPR-Cas system remains unknown.

In **Chapter 3 – 5** we were able to link *C. jejuni* ganglioside mimic expression to increased intestinal epithelial cell binding, endocytosis-mediated invasion and translocation. A ques-

tion that needs to be answered from these studies is which eukaryotic cell receptor binds ganglioside mimic structures enabling its enhanced virulence. Next, at the end stage of endo-lysosomal fusion ganglioside mimic positive isolates seem to form large compartments containing multiple *C. jejuni* bacteria. Is this beneficial for *C. jejuni* survival? What is more a mystery is which eukaryotic and *C. jejuni* proteins are involved in enabling *C. jejuni* exocytosis from the endo-lysosomal compartment to the basolateral intestinal epithelial surface to enter the deeper layers of the intestine. Unfortunately, lack of an animal model mirroring the human situation also disables us to extrapolate the *in vitro* observations and mechanisms to an *in vivo* situation. Leaving unanswered whether or not *in vitro* findings remain valid *in vivo*.

In **Chapter 6** the low prevalence of the large plasmids pVir and pTet in The Netherlands, might be related to the CRISPR-Cas system as discussed in **Chapter 2.** In the latter we show that a CRISPR spacer from different *C. jejuni* isolates has 96% identity with the pVir plasmid. It remains to be elucidated whether the CRISPR-Cas system is responsible for the low prevalence of these two plasmids in The Netherlands compared to other geographical regions.

Chapter 7 demonstrates that if a new GBS trial is started we not only need to focus on *C. jejuni* alone, but also on other *Campylobacter* species, such as *C. coli*. This would not only enable us to answer whether *C. coli* or other *Campylobacter* species play a role in the development of GBS, but also whether ganglioside mimic expression by these bacterial species is associated with GBS induction.

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Chapter 9

English summary

SUMMARY

Campylobacter jejuni is a major cause of diarrheal disease in man and is associated with post-infectious complications, such as the Guillain-Barré Syndrome (GBS). Sugar structures on the outer membrane of a subset of *C. jejuni* isolates were found to mimic sugar structures on the outer membrane of human peripheral nerve cells. The *C. jejuni* ganglioside mimics are only generated when the genes *cst*-II or *cst*-III are present. So far, the *cst*-II gene is the only functionally relevant microbial GBS marker in *C. jejuni*. In a small percentage of *C. jejuni* diseased patients, antibodies will be generated against the *C. jejuni* ganglioside mimics, most likely contributing to the induction of GBS. However, at least 50% of the GBS patients have generated ganglioside mimic antibodies that do not precisely match the ganglioside mimics expressed on the corresponding bacterial isolate. Why only some patients develop such antibodies and others not remains obscure. Earlier, ganglioside mimics were found to affect serum resistance of *C. jejuni*. For other bacteria, such as *N. meningitidis* and *H. influenzae* it has been established that ganglioside mimics are involved in virulence and in bacteriophage defense, next to serum resistance.

The specific aim of this thesis was to improve our insight in the features of ganglioside mimics in *C. jejuni* pathogenesis; this to enhance our knowledge on why a subset of patients infected with *C. jejuni* develop GBS. A second aim of this thesis was to identify new GBS markers in *C. jejuni*, since ganglioside mimics alone do not seem to be the only factor involved in the induction of GBS. A third aim of this thesis was to address the role of *C. coli* in GBS.

In our search for novel *C. jejuni* GBS markers, we focused on the CRISPR-Cas system, an adaptive immune system in bacteria and archaea. CRISPR-Cas is involved in mobile DNA neutralization with the CRISPR spacers being transcribed, processed and used for bacteriophage defense by Cas-protein guided RNA interference. We hypothesized that CRISPR-Cas spacers might select for presence or absence of the horizontal gene transfer (HGT) exchangeable LOS biosynthesis gene loci harboring the GBS marker *cst*-II in *C. jejuni*. After analysis, we only observed mutations in the CRISPR-associated genes that specifically correlated with GBS only, and/or with the presence/absence of the GBS marker *cst*-II. As the CRISPR-Cas system is shown to be involved in bacteriophage defense we analyzed if the observed mutations affected bacteriophage resistance. We revealed that the GBS isolates were less susceptible to bacteriophage infections compared to the enteritis isolates. More specifically, we found that bacteriophage resistance coincided with the presence of the GBS-associated ganglioside mimics. We could confirm this finding by generation of sialyltransferase (Cst-II) knock-out mutants lacking ganglioside mimic expression. A complemented *cst*-II mutant with partially restored ganglioside mimic expression established our finding that GBS-associated ganglioside mimics serve as a bacteriophage defense system. Remarkably, knock-out mutagenesis of one of the *cas* genes *csn1* revealed only a minor role of the CRISPR-Cas system in bacteriophage defense in the GBS isolates. In contrast, in an enteritis isolate the CRISPR-Cas system

was found to be of more importance in bacteriophage defense. These results corroborate that GBS-associated ganglioside mimics affect CRISPR-Cas function in bacteriophage defense. As the *cas* gene *csn1* is only found in vertebrate-associated pathogens, we addressed whether this gene evolved in the GBS isolates to a different function instead of a role in bacteriophage defense. Using *in vitro* assays we discovered that *cas* gene *csn1* of the GBS isolates evolved more towards a role in virulence. Next to that, we observed a relation between *csn1* and binding of IgG serum antibodies to the sialylated LOS structures of the GBS isolates. We thus show for the first time in *C. jejuni* that there is a distinct link between viral defense, virulence and GBS in a pathogenic bacterial species.

As discussed earlier, microbial LOS structures have been implicated in microbial pathogenicity. For *C. jejuni* little information was available on the involvement of (sialylated) LOS in bacterial pathogenicity. In this thesis, we established that the presence of ganglioside mimics (sialylated LOS) significantly increased the invasion potential of both human- and chicken-derived *C. jejuni* isolates. Inactivation and complementation of the gene sialyltransferase (Cst-II) established the role of ganglioside mimics in invasion. Next, we found enhanced cellular binding of *C. jejuni* isolates expressing ganglioside mimics as compared to *C. jejuni* isolates lacking ganglioside mimics. *C. jejuni* isolates with ganglioside mimics showing increased cellular binding also revealed enhanced co-localization with the early endosomal markers EEA1, Rab5 and the late endosomal marker LAMP-1, reflecting increased cellular endocytosis. Both *C. jejuni* isolates expressing or lacking ganglioside mimics were finally found to reside within acidic endo-lysosomal vesicles. The enhanced cellular endocytosis by ganglioside mimic-expressing *C. jejuni* isolates did promote increased survival, numbers of translocating bacteria and elevated epithelial release of the T-cell attractant CXCL10. These results are of interest, since *C. jejuni* with ganglioside mimics are often associated with the induction of severe gastro-enteritis and bloody stools. Next to ganglioside mimics, also the virulence plasmid pVir is associated with *C. jejuni* induced bloody stools in a Canadian study. Remarkably, in contrast to this Canadian study we did not find an association between the virulence plasmid pVir, bloody stools in The Netherlands, neither could we link the presence of pVir in *C. jejuni* with GBS

In addition to *C. jejuni*, other *Campylobacter* species have been isolated from GBS patients. In a study presented here in this thesis, we isolated from each of two GBS patients a *C. coli* isolate. For both isolates we could not link ganglioside mimics with GBS inductions, although one *C. coli* isolate did express sialylated LOS. Interestingly, the GBS patient that harbored the *C. coli* isolate without ganglioside mimic structures did contain anti-ganglioside mimic antibodies in her serum.

In conclusion, in this thesis we demonstrated the importance of GBS-associated ganglioside mimics for two other aspects of *C. jejuni*, in addition to serum resistance. GBS-associated ganglioside mimics provide protection against bacteriophage infections and promote virulence of both chicken- and human-derived *C. jejuni* isolates. Presence of the GBS marker *cst*-II,

correlated with mutations in a second bacteriophage defense system, CRISPR-Cas and was found to be important for *C. jejuni* virulence. Next to that, in the GBS-associated isolates we established that the *cas* gene *csn1* was needed for full virulence. Importantly, *csn1* seems to be linked with the generation of antibodies against the sialylated LOS structures of the GBS isolates, indicative for a biologically relevant role in membrane topology or composition. Secondly, we were unable to link the plasmid pVir with bloody stools and GBS induction in The Netherlands. Finally, other *Campylobacter* species can be isolated from GBS patients, although it remains to be elucidated whether or not ganglioside mimics of these species have a function in GBS induction. Overall, our results suggest that ganglioside mimics alone are not the only causative factor for GBS.

Chapter 10

Nederlandse samenvatting

SAMENVATTING

Campylobacter jejuni is een van de belangrijkste veroorzakers van diarree en wordt daarnaast geassocieerd met post-infectieuze complicaties, zoals het Guillain-Barré Syndroom (GBS). Suikerstructuren op het buitenmembraan van een subgroep van *C. jejuni* isolaten blijken identiek te zijn aan de structuren aanwezig op het membraan van humane perifere zenuwcellen, ook wel gangliosides genaamd. De ganglioside mimics op het *C. jejuni* membraan kunnen alleen gemaakt worden wanneer het sialyltransferase gen *cst*-II of *cst*-III aanwezig is. Belangrijk om op te merken, het *cst*-II gen tot nu toe de enige geïdentificeerde GBS marker.

Om tot nu toe voor ons onbekende redenen genereren een subgroep van *C. jejuni* geïnfecteerde patiënten antilichamen die zowel gericht zijn tegen de *C. jejuni* ganglioside mimics, maar ook tegen de gangliosides aanwezig op de zenuwcel. Er lijkt dus in deze patiënten auto-immuniteit te worden geïnduceerd. Door kruisreactiviteit van deze antilichamen met de menselijke ganglioside structuren op de zenuwcel kan dit uiteindelijk resulteren in immuun gemedieerde schade aan de perifere zenuwen. Schade aan de perifere zenuwcellen is de veroorzaker van de post-infectieuze complicatie GBS. Echter, een bijkomende moeilijkheid in het GBS onderzoek is dat minimaal 50% van deze anti-ganglioside mimic antilichamen in de GBS patiënten niet overeenkomt met de ganglioside mimics aanwezig op de corresponderende *C. jejuni* bacteriestam. Waarom sommige patiënten deze antilichamen dus ontwikkelen en de meeste andere *C. jejuni* geïnfecteerde patiënten niet blijft helaas erg onduidelijk.

Voor een aantal andere bacteriesoorten (*Neisseria meningitidis* en *Haemophilus influenzae*) is er experimenteel bewijs gegenereerd dat ganglioside mimics naast serum resistentie en hun mogelijke associatie met GBS ook betrokken zijn bij virulentie en faagresistentie.

Het doel van het onderzoek beschreven in dit proefschrift was dan ook om beter inzicht te verkrijgen in de specifieke functie(s) van de *C. jejuni* ganglioside mimics. De opgedane kennis kan er dan uiteindelijk toe bijdragen de vraag te beantwoorden waarom een subgroep van patiënten besmet met de bacterie *C. jejuni* GBS ontwikkeld. Een tweede doel van dit proefschrift was erop gericht om nieuwe *C. jejuni* GBS markers te identificeren. Er zijn namelijk naar ons idee sterke aanwijzingen in de literatuur dat de *C. jejuni* ganglioside mimics alleen niet de enige bacteriële factor is voor de inductie van GBS. Een derde doel van het proefschrift was om inzicht te verkrijgen of *C. coli* een rol zou kunnen spelen in GBS.

Omdat de GBS marker *cst*-II tesamen met een LOS biosynthese gen cluster uitwisselbaar is tussen *C. jejuni* stammen viel ons oog in onze zoektocht naar nieuwe GBS markers al gauw op een adaptief bacterieel immuunsysteem, CRISPR-Cas genaamd. CRISPR-Cas is in bacteriën en archaea erg belangrijk voor bescherming tegen virussen of ander vreemd DNA. Door de uitwisselbaarheid van o.a. het *cst*-II gen was onze hypothese dat er eventueel markers aanwezig zouden kunnen zijn in het CRISPR-Cas systeem die associëren met de aanwezigheid van o.a. het *cst*-II gen. En inderdaad, dit systeem bleek mutaties te bevatten, die associeerden met GBS alleen (nieuwe specifieke GBS markers) of met de aanwezigheid van de eerder ontdekte

GBS marker *cst*-II. Omdat het CRISPR-Cas systeem betrokken is bij o.a. bacteriofaagafweer, vroegen we ons ook af of deze mutaties van invloed zouden kunnen zijn op diezelfde bacteriofaagafweer. In de GBS stammen bleek het effect van deze mutaties echter gering. Echter de enteritis stammen waren daarentegen over het algemeen vrij gevoelig voor bacteriofaaginfecties. Bij deze analyse viel het op dat de faag gevoeligheid mede bepaald leek te worden door aanwezigheid van de GBS geassocieerde ganglioside mimics. Doormiddel van sialyltransferase (*cst*-II) knock-out mutanten die defect zijn in ganglioside mimic expressie en een *cst*-II complementant waarin de ganglioside mimic expressie weer hersteld was, konden we aantonen dat ganglioside mimics inderdaad een belangrijke rol spelen in bacteriofaagafweer. Door knock-out mutagenese van een van de CRISPR-Cas genen, het *csn1* gen, konden we aantonen dat dit systeem in GBS isolaten niet echt betrokken was bij bacteriofaagafweer, maar wel in een enteritis isolaat. Met *in vitro* essays ontdekte we dat in de GBS isolaten het *csn1* gen een andere belangrijke rol vervulde, namelijk in virulentie. Daarnaast lijken onze resultaten er op te wijzen dat het *csn1* gen een rol speelt in de immune response die GBS patiënten genereren, gericht tegen het gesialyleerde LOS van *C. jejuni*. We laten dus voor het eerst in *C. jejuni* zien dat er een verband bestaat tussen virale afweersystemen, virulentie en GBS.

Zoals eerder aangegeven, microbiële LOS structuren zijn een belangrijke virulentie factor en spelen daarnaast een rol in serumresistentie. Voor *C. jejuni* was hierover nog weinig bekend, maar in dit proefschrift hebben wij nu vastgesteld dat de aanwezigheid van ganglioside mimics (gesialyleerde LOS structuren) niet alleen een belangrijke rol vervullen in bacteriofaagafweer, maar ook in epitheliale invasie. Deze resultaten konden we verder bevestigen met behulp van de eerder beschreven *cst*-II knock-out mutanten en een *cst*-II complementant. Vervolgens konden we door opnieuw gebruik te maken van epitheliale cellen vaststellen dat *C. jejuni* isolaten met ganglioside mimics ten opzichte van *C. jejuni* isolaten zonder ganglioside mimics beter konden binden aan deze cellen. De consequentie van deze verbeterde binding was dat de *C. jejuni* isolaten met ganglioside mimics gemakkelijker epitheel cel binnen kwamen via cellulaire endocytosis. Dit toonden we aan door co-lokalisatie kleuringen met de endosomale markers EEA1, Rab5 en LAMP-1. Intracellulaire *C. jejuni* bacteriën, met dan wel zonder ganglioside mimics, vonden we uiteindelijk allebei terug in endo-lysosomale compartimenten. Deze compartimenten zijn een plek in de cel waar grote moleculen, zoals suikers, vetten, maar ook bacteriën verteerd kunnen worden. Gebruikmakend van een overlevingsessay, beschreven door Watson en Galan, konden we aantonen dat maar een klein percentage van de *C. jejuni* isolaten uiteindelijk in staat was om in dit compartiment te overleven. De *C. jejuni* stammen met ganglioside mimics hadden daarbij het voordeel dat hun mogelijkheid om binnen te komen in grotere aantallen uiteindelijk leidde tot meer overlevende bacteriën. Deze observatie viel samen met een verhoogde epitheliale uitscheiding van de T-cel marker CXCL10 en aantallen *C. jejuni* bacteriën aan de basolaterale zijde van een *in vitro* gecreëerde darm epitheel barrière. In andere woorden, ganglioside mimics lijken

uiteindelijk *C. jejuni* translocatie door darmepitheel te faciliteren. Het interessante is dat *C. jejuni* isolaten met ganglioside mimics vaker ernstige gastro-enteritis en bloederige diarree induceren in de mens. Het zou dus best zo kunnen zijn dat dit ernstiger ziekteverloop wordt geïnduceerd, doordat ook *in vivo* de ganglioside mimics de translocatie van *C. jejuni* door de darm faciliteren. Dit verdient dus nader onderzoek.

In de literatuur zijn niet alleen *C. jejuni* bacteriën die ganglioside mimics tot expressie brengen in verband gebracht met ernstige gastro-enteritis en bloederige diarree. Een associatie met dit type ziekteverloop is ook gelegd met de aanwezigheid van het pVir plasmide in *C. jejuni*. In tegenstelling tot een eerder onderzoek in Canada, hebben wij geen associatie kunnen vinden tussen de aanwezigheid van het virulentie plasmide pVir en bloederige diarree in Nederland. Ook vonden we geen verband tussen de aanwezigheid van dit plasmide en GBS.

Niet alleen *C. jejuni*, maar ook andere *Campylobacter* soorten worden in verband gebracht met GBS. Vanuit twee GBS patiënten isoleerde onze groep uit beide patiënten een *C. coli* stam. Een van de twee *C. coli* isolaten bracht niet de GBS geassocieerde ganglioside mimics tot expressie. Echter, in de corresponderende GBS patiënt werden wel anti-ganglioside mimic antilichamen gedetecteerd in het serum.

Concluderend, in dit proefschrift hebben we aangetoond dat de ganglioside mimics aanwezig op een subgroep van *C. jejuni* isolaten twee andere functies vervullen naast serum resistentie. Ganglioside mimics geven als eerste bescherming tegen bacteriofagen, maar beïnvloeden ook de virulentie van zowel uit kippen als uit mensen geïsoleerde *C. jejuni* isolaten. Daarnaast valt de aanwezigheid van ganglioside mimics samen met mutaties in een recent ontdekte bacteriofaag afweersysteem, CRISPR-Cas. Het CRISPR-Cas systeem biedt in *C. jejuni* niet alleen bescherming tegen bacteriofagen, maar is in voornamelijk de GBS isolaten ook geëvolueerd naar een essentiële rol in virulentie. Daarnaast bleek *csn1* een rol te spelen in de antilichaam respons tegen gesialyleerde suikerstructuren. Dit zou er op kunnen wijzen dat *csn1* betrokken is in membraan topologie of compositie. We konden geen verband leggen tussen pVir, bloederige diarree en GBS. Als laatste toonde we aan dat ook andere *Campylobacter* soorten met GBS in verband kunnen worden gebracht. Echter of dat bij deze soorten de ganglioside mimics ook een rol spelen in de inductie van GBS, verdiend nog nader onderzoek. Gebaseerd op deze en andere resultaten in dit proefschrift kunnen we dus stellen dat ganglioside mimics alleen niet de enige bacteriële factor is verantwoordelijk voor het ontstaan van GBS.

Appendices

List of Abbreviations
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List of Abbreviations

LIST OF ABBREVIATIONS

GBS	Guillain-Barré Syndrome
LOS	Lipo-oligosaccharides
GM	Ganglioside mimicry
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
cas	CRISPR associated genes
***cst*-II**	sialyltransferase
C. jejuni	*Campylobacter jejuni*
N. meningitidis	*Neisseria meningitidis*
H. influenzae	*Haemophilus influenzae*
W. succinogenes	*Wolinella succinogenes*
MFS	Miller Fisher Syndrome
EEA1	Early endosomal antigen 1
LAMP-1	Lysosomal-associated membrane protein 1
Rab5	mammalian small GTPase
Rab7	mammalian small GTPase
MLST	Multilocus sequence typing
PFGE	Pulsed Field Gel Electrophoresis
AFLP	Amplified Fragment Length Polymorphism
RFLP	Restriction Fragment Length Polymorphism
NCTC	National Collection of Type Cultures
TEER	Trans Epithelial Electric Resistance
LDH	Lactate Dehydrogenase
CFU	Colony Forming Units
RA	Reactive Arthritis
MOI	Multiplicity of Infection
CC	Clonal Complexes
MIC	Minimal Inhibitory Concentration

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37. Arjan R. Jansz, Drs.
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Curriculum Vitae

Rogier Petrus Leonardus Louwen was born on March 21st, 1977 in Schiedam, The Netherlands. In 1993 he finished his secondary school education (MAVO) at the RK MAVO Dommelbergen in Oosterhout. In 1995 he finished his secondary school education (HAVO) at MGR. Frencken College in Oosterhout, and began further education at the Hogeschool Brabant in Etten-Leur, studying 'Medical Laboratory Techniques' with specialization Microbiology. In 1999-2000 he completed his bachelor internship at The Scripps Research Institute, San Diego (USA) in the Molecular Biology laboratorium under supervision of Prof. Dr. Peter Wright, Assistant Prof. Jane Dyson and Dr.Ir. Gerard Kroon on the project "cloning, expression and purification of scFv fragments of the catalytic antibodies 33F12 and 43C9" for NMR studies. After graduating in 2000, he started working at the Erasmus MC as a Research Technician under supervision of Prof.Dr. Frank Grosveld, Drs. Maarten Sinaasappel and Dr. Bob Scholte on the project "Gene therapy for Criggler Najjar patients". In 2001 he became Board member of the SP department in Oosterhout. From 2001 – 2005 he followed different SP trainingships, including management, debating and financial trainings. Seminars and workshops were also followed under supervision of different SP trainers, including Jan Marijnissen. At the end of 2003 he started working as a Research Technician at the Department of Medical Microbiology and Infectious Diseases at the Erasmus MC on the Human Frontier Science Project (HFSP), studying the association between *Campylobacter jejuni* and the Guillain-Barré Syndrome. In 2006 he was allowed to start his PhD training on this subject at the Department of Medical Microbiology and Infectious Diseases. In 2005 he joined the PvdA department in Oosterhout and became member of the organization committee PvdA health care debate October 2005. In 2006 he was elected as a city council member for the PvdA department in Oosterhout. As a PhD student he was given a Yakult poster award at the NVMM 2006 spring congress. At the year 2007 he became member of the organization committee youth debate, which was organized in February of that year. Rogier wrote columns in the newspaper BN de Stem for this youth debate and in that same year he became secretary for the local PvdA department Oosterhout. From 2006 – 2010 he followed different PvdA and city council member trainings; including financial, management, civil planning and debating courses.

From 2004 – 2011 he was at different occasions speaker and poster presenter at different congresses. In 2009 he obtained a travel grant from the CHRO to join the CHRO2009 congress in Niigata (Japan). At that congress he became a scientific award winner "*Campylobacter* Research Award" for his presentation entitled "The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) related *cas* gene *Cj*1523 influences the virulence potential of *Campylobacter jejuni* and is associated with the Guillain-Barré Syndrome". In 2011 he was asked to become webmaster of the Department of Medical Microbiology and Infectious Diseases and followed Morello software training for Webmasters. In 2011 he was elected as Board member of the local PvdA department Oosterhout with as responsibility Treasurer. In 2011 he joined the editorial board of the WJCID for a four year period. For the NVMM congress

in April 2012 he is organizing, with Dr. Peter van Baarlen his co-promotor and Dr. Arnoud van Vliet a CRISPR-Cas session. Rogier is married to Marisja and together they have two daughters Cheyenne and Nikita. Rogier is currently continuing his research at the Department of Medical Microbiology and Infectious Diseases, Erasmus MC, The Netherlands.

List of publications

LIST OF PUBLICATIONS

van der Wegen P, **Louwen R,** Imam A, Buijs-Offerman R, Sinaasappel M, Grosveld F and Scholte BJ.

Successful treatment of UGT1A1 deficiency in a rat model of Crigler-Najjar disease by intravenous administration of a liver-specific lentiviral vector.

Mol. Ther. 2006 Feb;13(2):374-81

Louwen R, van Belkum A, Wagenaar J, Doorduyn Y, Achterberg R and Endtz H.

Lack of association between the presence of the pVir plasmid and bloody diarrhea in *Campylobacter Jejuni* enteritis.

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Louwen R, Heikema A, van Belkum A, Ott A, Gilbert M, Ang W, Endtz H, Bergman M and Nieuwenhuis E.

The sialylated lipo-oligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion.

Infect Immun. 2008 Oct;76(10):4431-8.

van Belkum A, Jacobs B, van Beek E, **Louwen R,** van Rijs W, Debruyne L, Gilbert M, Li J, Jansz A, Mégraud F and Endtz H

Can *Campylobacter coli* induce Guillain-Barré Syndrome?

Eur J Clin Microbiol Infect Dis. 2008 Nov 12.

Habib I, **Louwen R,** Uyttendaele M, Houf K, Vandenberg O, Nieuwenhuis E, Miller W, van Belkum A, and de Zutter L.

Correlation between genotypic diversity, lipo-oligosaccharide gene locus class variation, and Caco-2 Cell invasion potential of *Campylobacter jejuni* isolates from chicken meat and humans: contribution to virulotyping.

Appl Environ Microbiol. 2009 Jul;75(13):4277-88.

Louwen R, Nieuwenhuis E, van Marrewijk L, Horst-Kreft D, de Ruiter L, Heikema A, van Wamel W, Wagenaar J, Endtz H, Samsom J, van Baarlen P, Akhmanova A and van Belkum A.

Campylobacter jejuni translocation across intestinal epithelial cells is facilitated by ganglioside-like lipooligosaccharide structures.

In preparation for resubmission to Infection and Immunity 2012

Louwen R, Horst-Kreft D, de Boer A, van der Graaf - van Bloois L, Heikema A, van Putten J, de Knegt G, Hamersma M, Timms A, van Wamel W, Endtz H, van der Oost J, Wagenaar J, Wells J, van Vliet A, Willemsen P, van Baarlen P, Nieuwenhuis E and van Belkum A.

A distinct link between virulence and viral defense in a pathogenic bacterial species.

In preparation for Current Biology 2012

Louwen R, van Baarlen P, van Vliet A, van Belkum A, Hays J.P. and Endtz H.P.

Review *Campylobacter* bacteremia: a rare and underreported event?

Submitted to the European Journal of Microbiology and Immunology 2012

PhD portfolio

PHD PORTFOLIO

Name PhD student:	Rogier P.L. Louwen
Erasmus MC department:	Medical Microbiology and Infectious Diseases
PhD period:	June 2006 – June 2011
Promotor:	Prof.dr.dr. Alex van Belkum
Promotor:	Prof.dr. Edward E. S. Nieuwenhuis
Co-Promotor:	Dr.Ir. Peter van Baarlen

National and international conferences	**Year**	**ECTS**
• Scientific Autumn Meeting NVMM (oral presentation)	2004	0.5
• Scientific Meeting NVGE (oral presentation)	2004	0.5
• Scientific Spring Meeting NVMM (poster presentation)	2006	1
• Scientific Meeting NVVI (poster presentation)	2006	0.5
• CHRO Netherlands Rotterdam (two poster presentation)	2007	2
• Scientific Spring Meeting NVMM (oral presentation)	2008	0.5
• CHRO Japan *Campylobacter*/*Helicobacter* congress (oral presentation)	2009	0.5
• *Campylobacter jejuni* UK meeting 2009 (oral presentation)	2009	0.5
• CRISPR/Cas congress Wageningen (oral presentation)	2010	0.5
• CampyGerm congress Göttingen Duitsland (oral presentation)	2011	0.5
• Organizing CRISPR-Cas Session Spring Meeting NVMM	2011-2012	0.5
Scientific meetings		
• HFSP meeting Ottowa Canada (oral presentation)	2004	2
• Departmental Research Days (oral presentations)	2008-2010	2
Seminars and Workshops		
• Departmental Journal Clubs	2006-2010	1
• Departmental Research Meetings	2004-2010	6
• Pediatrics departmental Research Meetings	2004-2010	6
• GBS work meetings Rotterdam	2004-2011	0.5
• Immunity in the central nervous system Symposium	2005	1
• Guillain-Barré Syndrome Symposium	2006	1
• Mucosal Immunology Symposium	2007	1
Grants		
• Travel grant CHRO2009 (Niigata)	2009	0.5
Scientific Awards		
• Yakult Poster price NVMM	2006	
• *Campylobacter* Research Award CHRO2009 Japan	2009	
Specific Courses		
• Molecular Medicine Postgraduate School Molecular Immunology	2006	1.5
• Erasmus MC Optical Imaging Centre Introduction course	2008	0.5
Zeiss Confocal microscopy		

Teaching

Supervision of students

• Supervison of Bachelor of Science students	2004-2010	3
• Supervision of medical students "Vaardigheidsonderwijs Infectieziekten"	2006-2010	3
• Lectures for masterclass students (Summercourse and Wintercourse)	2009-2011	2

Dankwoord

DANKWOORD

Zo, de hobbelige promotieweg (traject) is gefietst! Vele frustraties van het mislukken van proeven (labjournaals vol), leverde "meestal" weer een glimlach op, wanneer deze hoge bergen waren beklommen en het uitzicht uiteindelijk toch mooi bleek te zijn. Dit ging echter natuurlijk niet vanzelf en heeft veel hulp en ondersteunende woorden gekost van niet alleen begeleiders, collega's, maar ook van familie en vrienden. Promoveren is dus eigenlijk als wielrennen, je hebt een ploegleider, coaches, masseurs, ondersteunende crew, de supporters, politie, ambulance en de rondemissen. Tijdens het schrijven van dit proefschrift dringt het pas daadwerkelijk tot je door dat een promovendus zonder teamwork en medische hulp de eindstreep nooit zal halen. Via deze weg wil ik daarom mijn dankwoord aan al deze mensen individueel uitspreken die ervoor gezorgd hebben dat deze wielrenner met "vallen" en opstaan uiteindelijk toch de finish heeft gehaald.

Ploegleider Alex van Belkum, Zoals Edwin Evers zou zeggen een typisch gevalletje open deur. Ja, **Alex** jouw deur in café van Belkum stond altijd open. Zowel voor de momenten dat ik weer overenthousiast naar binnen kwam rennen. Gelukkig ook voor de keren die vaak daarna volgde dat ik met een gebogen hoofd moest komen vertellen dat de *p* waarde toch niet zo ….. was. Volgens mij moet het bier in jou café "van Belkum" op onze afdeling erg goed zijn geweest, want hierover werd je nooit boos (waarschijnlijk heb je er wel heel veel binnenpretjes over gehad). Daarnaast heb ik het als zeer prettig ervaren dat als er moeilijke momenten waren in de privésfeer je deur ook altijd open stond voor een hart-lucht-moment. Ook je telefoontjes met de vraag hoe het met me ging, nadat ik met fiets en al was geschept door een auto heb ik gewaardeerd. Alex bedankt dus voor alle hulp, met zowel het snelle corrigeer werk van de artikelen en het tot stand komen van dit proefschrift tot het meer socialere. Hopelijk houden we nog even contact!

Coach Edward, vrij snel maakte ik ook kennis met jou, toen ik begon aan het *Campylobacter* onderzoek. Toevallig zat je vlak naast de kamer van mijn oude baas Drs. Maarten Sinaasappel, waarschijnlijk heb je wel het een en ander van hem te horen gekregen (goede en misschien minder goede dingen) over mijn eerder werkprestatie op de celbiologie. Jouw enthousiasme en gedrevenheid waren een goede inspiratiebron voor mij. Ook al had je toen nog geen café, jouw deur stond ook altijd open voor werk gerelateerde vragen of hart-lucht-momenten. Als een echte coach heb je aan de juiste knoppen gedraaid om een aantal mooie artikelen voor elkaar te krijgen door me te begeleiden de verkregen resultaten wetenschappelijk op papier te zetten. De immunologie meetings hebben me extra scholing gegeven op immunologisch vlak, al moet ik toegeven dat ik ook regelmatig met flapperende oren heb gezeten, over alle immunologische termen die voorbij kwamen. Activiteiten buiten het kindergeneeskunde lab waren altijd gezellig, zoals o.a. de feestjes bij je thuis. Edward mijn dank is erg groot voor jouw bijdrage aan dit proefschrift en ik heb het erg gewaardeerd dat je na je vertrek naar een

topbaan in Utrecht je toch nog de tijd hebt genomen om met mij de laatste loodjes van dit proefschrift af te ronden.

September 2007 was het CHRO 2007 congres in Rotterdam, daar kennis gemaakt te hebben met jou (**coach Peter)** belanden we al snel samen met **Martijn Rolloos** op een terras tegenover het congrescentrum om even bij te tanken van alle presentaties. Het bier smaakte goed en al snel hadden we de eerste wilde theorie over maagcrophagen gecreëerd. Ja **Peter,** wie had dat gedacht, dat die dag het begin zou zijn van een zeer goede wetenschappelijke samenwerking en een erg leuke en gezellige vriendschap die ik zeer koester. Naast deze twee punten was je ook een zeer goede coach, wat altijd tot goede discussies leidde over het onderzoek maar wel met een goed glas bier op het terras in Wageningen. Ik ben je ook, net zoals aan Alex en Edward, zeer veel dank verschuldigd voor al het rode pen werk en alle hulp met het tot stand laten komen van drie zeer mooie gezamenlijke artikelen en dit proefschrift. Peter, ik hoop dat we onze wetenschappelijke discussies, wilde ideeën, bezoekjes aan Wageningen en onze samenwerking en vriendschap nog lang kunnen voortzetten. **Janneke,** jij kwam later bij de groep van Edward, maar bleek al snel een goede medecoach te zijn, die haarscherp het koren van het kaf kon scheiden met het bespreken van mijn resultaten tijdens de labmeetings, of met een basis te leggen voor een manuscript. Ook jou (immunologische) gedrevenheid en enthousiasme waren erg inspirerend. Je gemoedelijkheid en vriendelijkheid en rechtdoorzee mentaliteit heb ik zeer gewaardeerd, dank voor alle hulp en jouw luisterend oor voor niet alleen wetenschappelijke zaken.

Arnoud, na je vertrek naar Engeland (God save the Queen) hebben we een tijd geen contact gehad door de enorme plas water die er tussen ons en ons werk lag. Echter al snel bleek het CRISPR-Cas werk een goed bindmiddel kon zijn in gezamenlijk wetenschappelijk werk. Ook jij was een goede coach in het laatste stadium van mijn promotietraject, je hebt menig wilde ideeën van Peter en mij altijd met een realistisch oog bekeken en waar nodig ons gestimuleerd om er mee door te gaan, of toch maar geadviseerd om de wilde ideeën nog even in de ijskast te zetten. Dit heeft uiteindelijk geresulteerd naar mijn idee tot een van de mooiste werken in dit proefschrift, het CRISPR-Cas artikel, mijn dank hiervoor! Ik hoop dat ook onze samenwerking in de toekomst door zal blijven gaan, met wie weet nog wat CRISPR-Cas onderzoek in Engeland voor een paar maanden of langer. Arnoud bedankt!!

Masseuse Astrid, eigenlijk hoor je niet alleen bij de masseurs (oké, in jouw geval masseuse) thuis, maar ook bij de ondersteunende crew. Je was een goede masseuse (hoor je mijn lach, hehheh…) in die zin dat je altijd mijn manuscripten op een masserende manier nog net een niveau hoger wist te krijgen, met belangrijke detailaanpassingen. Vele proeven waren daarnaast niet mogelijk geweest als jij het niet voor elkaar had gekregen om de *cst*-II knock-outs en nog moeilijker een *Campylobacter cst*-II complementant te maken. Het woord complementant, is door ons nu een "forbidden word" geworden in het Campylobacter onderzoek. Ook veel dank voor het helpen van Gerjo om de *csn1* knock-outs te maken. Gelukkig was het niet alleen werk wat we samen deelde maar ook feestmomenten zoals Carnaval in

Oosterhout, of de dansvloer op de voorjaarsvergadering van de NVMM. Veel succes met het afronden van je eigen promotie onderzoek!! **Willem,** ook jij bedankt voor het masseren van de manuscripten met belangrijke tips, ideeën en detailaanpassingen om de manuscripten naar een hoger niveau te krijgen. **Peter** en **John,** raar hoe dingen toch kunnen lopen. De eerste jaren van mijn promotie waren jullie nog niet in beeld, maar het laatste half jaar tot jaar is jullie bijdrage aan mijn laatste manuscript van zeer belangrijke waarde geweest. Jullie manuscriptmassage heeft tot een zeer mooi artikel geleid, de kers op mijn proefschrift, mijn dank hiervoor. Daarnaast lijken onze wetenschappelijke interesses in fagen en CRISPR-Cas een mooie opstap te zijn voor verder gezamenlijk onderzoek en projectaanvragen. John en Peter, hopelijk vloeien hier nog vele mooie artikelen uit voort, met daarnaast ontspannende momenten op het terras van zowel Wageningen als Lelystad.

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