Aus dem Institut für Veterinär-Biochemie
und dem Institut für Lebensmittelhygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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"To think, analyse and invent are not anomalous acts, but the normal respiration of the intelligence."

Jorge Luis Borges

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List of abbreviations

C. jejuni	*Campylobacter jejuni*
E. coli	*Escherichia coli*
H. pylori	*Helicobacter pylori*
S. typhimurium	*Salmonella typhimurium*
Y. pseudotuberculosis	*Yersinia pseudotuberculosis*
APCs	antigen-presenting cells
B3GLCT	β1,3-glucosyltransferase
Cap	*Campylobacter* adhesion protein
CCV	*Campylobacter*-containing vacuole
CDC	United States Centre for Disease Control and Prevention
CDT	cytolethal distending toxin
CFU	colony forming units
CheY	chemotaxis protein Y
Cia	*Campylobacter* invasion antigens
CMP-NeuAc	CMP-*N*-acetylneuraminate
CPS	capsular polysaccharide
C1GalT1	Glycoprotein-*N*-acetylgalactosamine 3-β-galactosyltransferase 1
C2GnT/GCNT	β1,6-*N*-acetylglucosaminyltransferase
DCs	dendritic cells
ER	endoplasmic reticulum
EU	European Union
Flp	fibronectin-like protein
FUT8	fucosyltransferase 8
GalNAc	*N*-acetyl-D-galactosamine
GlcNAc	*N*-acetylglucosamine
GalNAc-T	UDP-GalNAc: polypeptide *N*-acetyl-D-galacosaminyl transferases
GALNT	*N*-acetyl-galactosaminyltransferase
GBS	Guillain-Barré syndrome
GI	gastrointestinal
GPA	gentamicin protection assay
G_2/M	growth pre-mitotic phase
HBGAs	human histo-blood group antigens
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells

IL	interleukin
IRAK	IL-1R-associated kinase
JlpA	surface-exposed lipoprotein
LOS	Lipoligosaccharide
LPS	Lipopolysaccharide
LTTR	LysR family transcriptional regulator
MAPK	Ras-mitogen-activated protein kinas
miRNA	MicroRNA
mRNA	Messenger RNA
MOMP	major outer membrane protein
ncRNA	Non-coding RNA
NLR	oligomerization domain-like receptors
NOD	nucleotide-binding oligomerization domain
ncRNA	Non-coding RNA
O-glycan	mucin type *O*-glycan
PAS	Per-Arnt-Sim motif
PEB1	periplasmic component of an aspartate/glutamate ABC-transporter
PRR	pattern recognition receptors
PTS	proline, threonine and serine
RM	restriction modification
RNAi	RNA interference
sLe^x	Sialyl Lewis X
STEC	Shiga toxin-producing Escherichia coli
ST3GAL	ST3 β-Galactoside α2,3-Sialyltransferase
ST6GAL	β-galactosidase α2,6 sialyltransferase
ST6GALNAc	ST6 *N*-acetyl-galactosaminide α2,6-sialyltransferase
ST8Sia	ST8 α-*N*-acetyl-neuraminide α2,8-sialyltransferase
SPDEF	Sam pointed domain containing Ets
TCS	two-component regulatory system
TLR	Toll-like receptor
TNF-α	tumour necrosis factor alpha
TFF	trefoil factor family
TRAF	TNFR-associated factor
T3SS	type-III secretion system
T4SS	type-IV secretion system
T6SS	type-VI secretion system

UC	ulcerative colitis
UTR	untranslated regions
WHO	World Health Organization
β3Gn-T6	β1, 3-N-acetylglucosaminetransferase 6

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1. General introduction

Campylobacter spp. are microaerophilic, gram-negative and motile bacteria belonging to the family of *Campylobacteraceae* (Bolton 2015). *Campylobacter jejuni* (*C. jejuni*) is the most frequent cause of bacterial food borne disease worldwide (Freitag, Strijbis, and van Putten 2017; Bouwman et al. 2014; Dasti et al. 2010). The most common symptoms caused by *C. jejuni* are manifested as gastroenteritis accompanied usually by fever, vomiting, abdominal pain and bloody diarrhoea (van Vliet and Ketley 2001; Bolton 2015). Despite the significant health burden caused by *C. jejuni* infections, our present knowledge about the interplay between *C. jejuni* and human hosts is still very limited.

C. jejuni has differentiated itself from many enteric bacterial pathogens, as it can persist in nature as a commensal microbe of many animal hosts yet promotes infection in human population (Burnham and Hendrixson 2018). Nowadays, with the availability of numerous complete genome sequences of various *C. jejuni* strains, our appreciation of genetics, physiology, pathogenesis and immunity of *C. jejuni* infections has greatly expanded (Backert et al. 2013). However, in contrast to other enteropathogens like *Salmonella*, *Listeria* and *Shigella*, the molecular basis of pathogenicity of *C. jejuni* in human still remains to be solved. This situation is partly due to the complexity and malleability of the biology of *C. jejuni*. Although the *C. jejuni* genome is much smaller than of other enteropahtogens, it has reserved genomic spaces to encode multiple and complex systems that mediate determinants required for *C. jejuni* infection. These systems include lipooligosaccharide (LOS) modification, protein glycosylation systems, multifunctional flagellum and multiple respiratory systems as well as transformation system (Burnham and Hendrixson 2018). The mechanism by which *C. jejuni* benefits from such complex systems is still unclear. Moreover, the intrastrain and the absence of typical bacterial adhesins, toxins or classical secretion systems in *C. jejuni* genomes make it particular challenging to study the pathogenicity of this pathogen.

C. jejuni predominantly colonise the mucus layer on the epithelium in the gastrointestinal (GI) tract and the mucus layer associated crypts of host. The mucus layer has sufficient nutrients and carbon sources to support *C. jejuni* metabolism and growth (Burnham and Hendrixson 2018). Previous studies using various animal and *in vitro* cell model systems indicated that the motility and chemotaxis are important for *C. jejuni* to thrive in the mucus milieu and subvert the mucus barrier and eventually colonise the GI tract (Backert et al. 2013). Moreover, processes like adhesion to, transmigration across, invasion into and intracellular survival within the host cells are important events promoting inflammation and disease development (Backert et al. 2013; Cróinín and Backert 2012). Both *O*- and *N*-linked glycosylation systems also play a

prominent role in host-pathogen crosstalk and disease outcome (Backert et al. 2013). *C. jejuni* encodes numerous determinants involved in these disease-associated processes and has evolved strategies to promote diseases in human hosts. Thus, identification of these virulence factors is the key for expanding our understanding of the molecular mechanisms of the pathogenesis and developing therapeutics to treat the infection.

The mucus barrier acting as a defence against commensal and invading pathogens is the first obstacle that *C. jejuni* encounters before attaching the epithelial cells. The secreted gel-forming mucin MUC2 as the major component of the mucus is considered as a dynamic responsive component of colonic mucus layer that interacts with and responds to the mucosal pathogens (Desai et al. 2016; Tu, McGuckin, and Mendz 2008). Upon pathogens infection, various factors such as microbial products, bacterial effectors and proinflammatory cytokines can induce mucin hypersecretion (Deplancke and Gaskins 2001; Navarro-Garcia et al. 2010; Xue et al. 2014). In addition, qualitative changes in mucin may occur upon pathogens infection, which is normally characterised by the alteration in degree of sulfation, sialylation and varied rate of glycosylation (Van Klinken et al. 1999; Linden et al. 2008; Kudelka et al. 2020). *C. jejuni* is able to modulate the expression and glycosylation of host mucins as well as their own glycan expression following infection to ensure a successful colonisation and invasion (Poole et al. 2018; Naughton et al. 2013). On the other hand, altered mucus glycosylation during infection can change the composition and structure of carbohydrates on the mucin surface, thereby affecting microbial adhesion as well as the capability of pathogens to degrade the mucins (Linden et al. 2008; Stone et al. 2010; Fu et al. 2011). The major components of the carbohydrates on cell surface proteins are oligosaccharide moieties, known as *O*-glycans (Poole et al. 2018; Jensen, Kolarich, and Packer 2010). *O*-glycans profoundly influence the pathogen-host interaction, as specific binding via “glycan-glycan talk” constitutes an important mechanism for bacteria to mediate adhesion and invasion of host cells (Poole et al. 2018). Previous studies demonstrated that the binding of *C. jejuni* to the host mucins is mediated by glycan-glycan interaction in the course of colonisation (Poole et al. 2018; Naughton et al. 2013). However, the knowledge about the mechanism of *C. jejuni* interaction with glycans of host mucosal surfaces and corresponding regulatory pathways involving mucin-modifying enzymes such as glycosyltransferases is still very limited.

The interaction of pathogen with mucus layer is a dynamic and complex processes that might be mediated by various factors. MicroRNAs (miRNAs) are a class of highly conserved endogenous that participate in physiological and pathological processes by regulating gene expression, such as inflammatory reactions, immune responses (Ma et al. 2020). They are reported to be the major regulator controlling glycan biosynthesis and modulating glycosylation

(Kasper, Koppolu, and Mahal 2014; Agrawal et al. 2014). Regulatory miRNA networks have been shown to play a significant role in the host response to the bacterial infection (Sharbati et al. 2011; Hoeke et al. 2013), which further underscore the necessity to identify a miRNA-mediated regulatory network that might contribute to the mucin regulation and modification during *C. jejuni* intestinal infection. Studies in this area would expand our knowledge of the mechanism by which *C. jejuni* infections modulate the host responses. A more complete understanding of the regulation of the host response mechanisms to the pathogenic challenge encountered during the infection would improve appropriate intervention strategies to cope with the *C. jejuni*-caused diseases.

2. Literature overview

2.1. *Campylobacter* spp.

2.1.1. Biology of *Campylobacter*

The taxonomic structure of *Campylobacter* has been a matter of controversy since its inception, as the taxonomic study of *Campylobacter* mostly depended on phenotypic character, which was difficult to standardise (On 2001). Owing to the application of advance taxonomic methods, the taxonomy of *Campylobacter* underwent an extensive rearrangement (On 2001). According to the latter report, the genus *Campylobacter* currently contains thirty-nine species and sixteen subspecies (Zenebe, Zegeye, and Eguale 2020). Most species of *Campylobacter* are animal, human or zoonotic pathogens (On 2013). Among them, *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli)* are the most important human pathogenic species, accounting for over 95% of campylobacteriosis in humans (Bolton 2015; Silva et al. 2011; Golz et al. 2014). *C. jejuni* are small (0.2-0.8 µm wide and 0.5-5 µm long), gram-negative, slender spirally curved rods that have a single polar unsheathed flagellum at one or both sides of the cell and thus exhibit a corkscrew-like motility (Bolton 2015; Silva et al. 2011). *C. jejuni* are oxidase active species and their energy source is amino acids or tricarboxylic acid cycle intermediates (Silva et al. 2011). *C. jejuni* are thermophilic and optimally grows at 37°C to 42°C, but incapable of growth below 30°C or above 55°C (Silva et al. 2011). Additionally, they are microaerophilic bacteria requiring 85% N_2, 10% CO_2 and 5% O_2 for optimal growth (Silva et al. 2011). These characteristics limit the ability of *C. jejuni* to multiply either outside of an animal host or in food during their processing and storage (Silva et al. 2011). Despite such restricted growth conditions, *C. jejuni* are ubiquitous in the environment and withstand various environmental stresses (Golz et al. 2014).

2.1.2. Epidemiology of *Campylobacter*

C. jejuni can live as commensal microbes in the gastrointestinal tract of many domestic animals and wild birds and mammals, yet it promotes infection in human intestinal tract to cause diarrhoeal disease (Golz et al. 2014; Bolton 2015). According to the latest report from the World Health Organization (WHO), *Campylobacter* is one of the four major global causes of foodborne bacterial diarrhoeal diseases (WHO 2020). As Community Zoonoses Reports from the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) published, since 2005, campylobacteriosis has been the most commonly reported gastrointestinal disease in humans in Europe Union (EU) followed by salmonellosis and Shiga toxin-producing *Escherichia coli* (STEC) infections (EFSA and ECDC 2019). In 2019, campylobacteriosis was the major zoonotic disease in humans with 220,682 confirmed cases reported in the EU (ECDC 2021). Comparing to the data from 2015 (229,213 cases)

(EFSA 2016), the trend of incident number of reported cases remained stable in the EU. In Germany, around 60,000-70,000 *Campylobacter* enteritis was reported with a notification rate of 70-90 per 100,000 populations in the year of 2017 to 2019 (RKI 2019, 2020). Incidence numbers in the last 15 years were summarised in Figure 1. Moreover, the Centre for Disease Control (CDC) reported that *C. jejuni* causes an estimated 1.5 million infections every year in the United States . According to the WHO, the reported infection incidence caused by *Campylobacter* was 5 million annually around the world (WHO 2019). As such, *C. jejuni* constitutes a potential threat to public health worldwide.

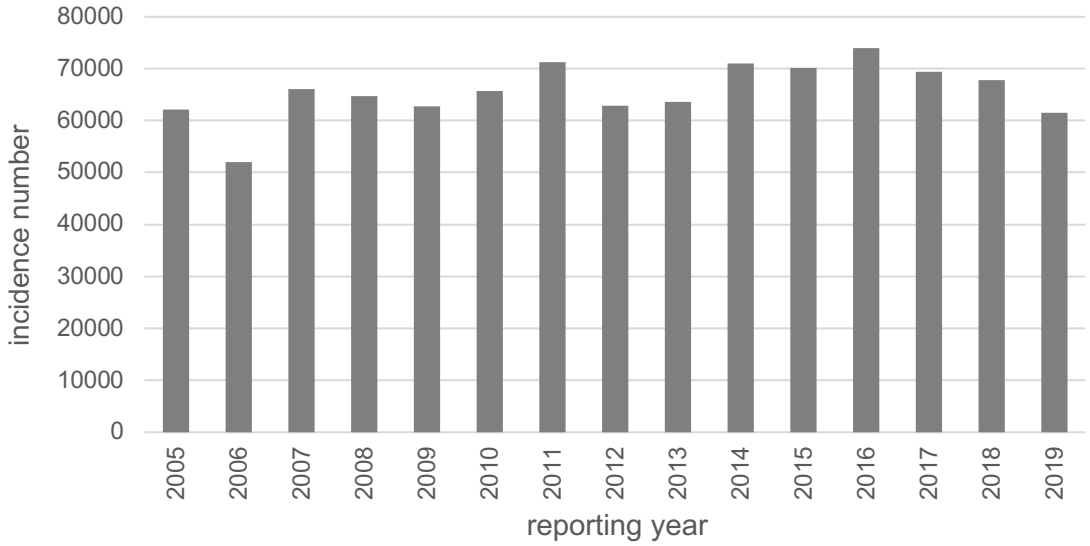

Figure 1. Number of *Campylobacter* enteritis transmitted by reporting year, Germany 2005–2019 (Robert Koch Institute, 2005-2020)

2.1.3. Transmission and post-infection sequelae of *Campylobacter*

C. jejuni is a commensal in avian species. It can colonise the intestines of chickens not causing overt disease. In humans, the major modes of transmission, as shown in Figure 2, are normally from consumption of infected poultry and cross-contamination with other food or water, which may contribute to more than 90% of all sporadic human cases (Golz et al. 2014). Nevertheless, contacting with pets or other animals and different environmental sources are considered for an adequate risk assessment (Golz et al. 2014).

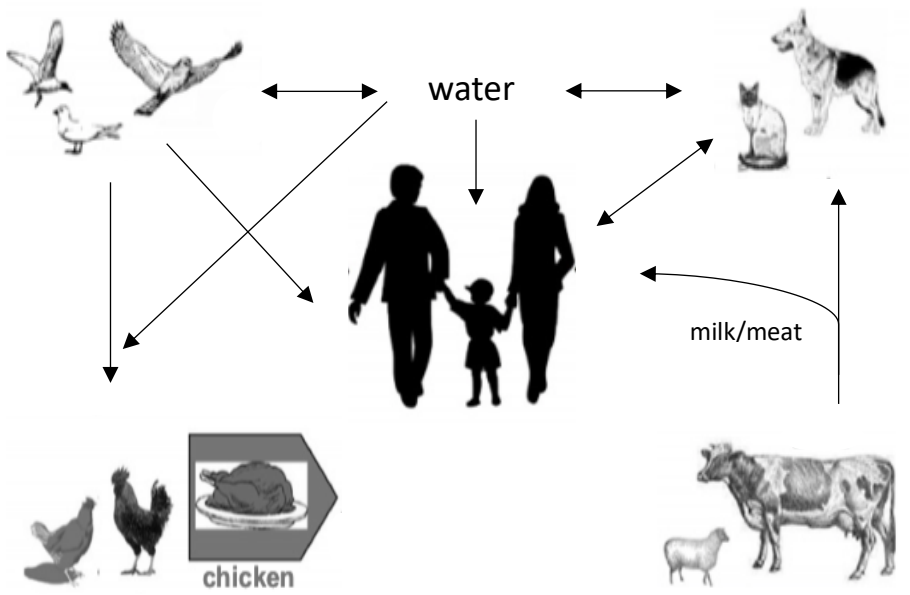

Figure 2. Most important routes for human infection by *Campylobacter jejuni*. This figure was reproduced from reference (Dasti et al. 2010).

Campylobacteriosis occurs at all ages with a preference in young infants (< 4 years) and young adults (20–29 years) (Golz et al. 2014). With a characteristic seasonality, *Campylobacter* is prone to be more prevalent in human during summer and early autumn (Fig. 3) (Golz et al. 2014; EFSA and ECDC 2019). These specific phenomena are possibly due to the age-determined differences in risk factors like mucosal structure and systemic immunity. Moreover, seasonal preference for food preparation as well as the age-specific and season-specific preferential patterns of exercises with higher exposure risk also contribute to the high incidence of campylobcateriosis in the warmer season (Golz et al. 2014; EFSA and ECDC 2019). Human infection needs an ingestion of at least 100-500 colony forming units (CFU) of *C. jejuni* to cause gastroenteritis (Alter et al. 2011). The lower sections of the intestinal tract including ileum, jejunum and colon are the niches *C. jejuni* prone to colonise (Dasti et al. 2010). In most symptomatic cases, campylobacteriosis is a self-limiting disease and manifest as 1–3 days of prodromal symptoms with fever, vomiting followed by 3-7 days of gastroenteritis associated with abdominal pain and watery, occasionally bloody diarrhoea (van Vliet and Ketley 2001; Bolton 2015; Dasti et al. 2010). In a minority of cases, the nerve disease Guillain-Barré syndrome (GBS) associated with paralysis can occur as 1 out of 1000 cases (van Vliet and Ketley 2001; Bolton 2015). Moreover, *Campylobacter* enteritis was one of the most common reportable diarrheal disease in the past decade that has been considered as an important risk factor for the development of inflammatory bowel disease (IBD) (Dasti et al. 2010).

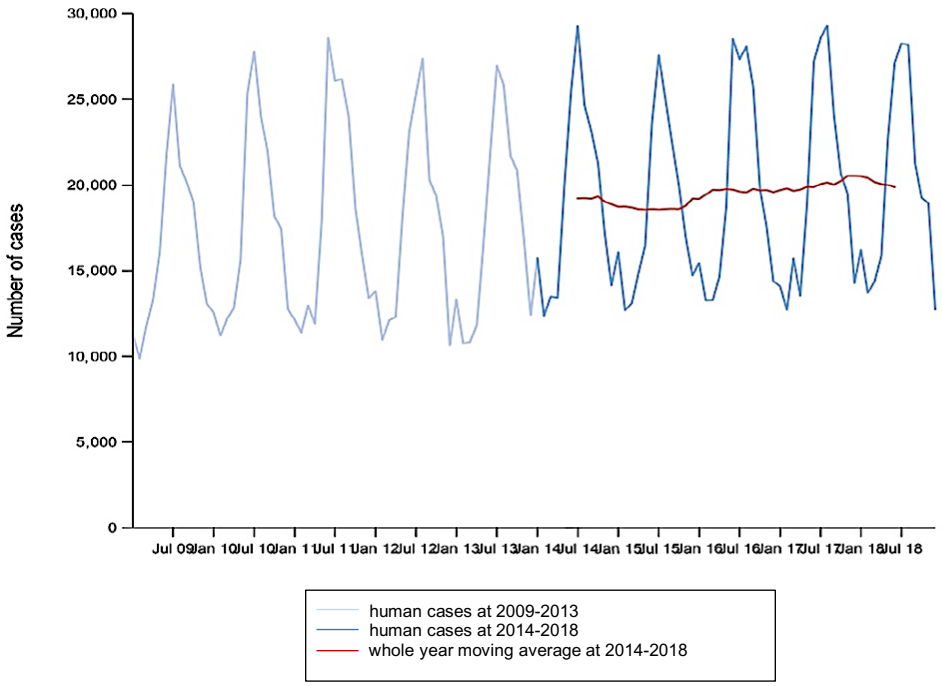

Figure 3. Trend in reported confirmed human cases of campylobacteriosis in the EU/EEA, by every January and July during 2009–2018. This figure was taken from reference (EFSA and ECDC 2019).

The last decade concerted efforts have been made to improve control measures and intervention strategies to minimize the occurrence of *Campylobacter* spp. in livestock and to decrease the germ burden in animal and food as well as to reduce the transmission along the food chain. However, as Figure 3 shown, a significant increasing trend in the number of confirmed campylobacteriosis cases has been reported in the EU over the period of 2009-2018, while a relative stable trend in the last five years from 2014 to 2018 (EFSA and ECDC 2019). Therefore, a combined effort from all aspect of, enhancing the on-farm biosecurity, improving hygienic slaughtering practices, comprehensive surveillance and raising public awareness, is urgently needed. Last but not least, a more complete understanding of not only the pathogenesis and survival mechanism of *Campylobacter* in environmental-, avian- and human hosts but also the mechanisms of host response to the diverse stages of pathogenesis of *Campylobacter* is required to facilitate appropriate intervention strategies to reduce the burden of *Campylobacter*-associated disease.

2.2. Research status of *Campylobacter jejuni* and models for studying *Campylobacter jejuni*

2.2.1. Research status of *Campylobacter jejuni* pathogenesis

C. jejuni is one of the most important foodborne bacterial. However, in contrast to many other enteric pathogens, our knowledge of how *C. jejuni* establishing infection and causing disease largely lags behind. Since 2000, Parkhill et al. (Parkhill et al. 2000) have completed the whole genome sequencing of *C. jejuni* NCTC 11168, recent efforts in decoding complete genome sequences from various *C. jejuni* isolates has greatly expanded our understanding in genetics, physiology, pathogenesis of *C. jejuni* infection (Backert et al. 2013). Numerous *in vitro* and *in vivo* studies have identified and characterised several *C. jejuni* virulence determinants and assessed the functional roles of related proteins by means of mutation of corresponding genes (Crushell et al. 2004). Based on the published reports thus far, adhesion, invasion, toxin production and subversion of host cell processes are considered as integral parts of virulence machinery of *C. jejuni* (Backert et al. 2013). Recent progress also advanced the knowledge of pathogenicity-associated processes like glycosylation and intracellular adaptation that play an essential role for *C. jejuni* colonisation and infection development (Cróinín and Backert 2012). Nevertheless, there is still a limitation in the complete understanding of *C. jejuni* pathogenesis thus far. This dilemma is partly due to the initial difficulties in genetic manipulations, natural heterogeneity of strains, variability of virulence in interstrain and conflicting data in the literature as well as scarcity of effective animal model of human enteric infection (Crushell et al. 2004).

2.2.2. Models used for studying *Campylobacter jejuni* pathogenesis

The lack of suitable experimental *in vivo* models has hampered the progress of understanding the molecular mechanism underlaying the immunopathology of *C. jejuni* (Bereswill et al. 2011). Mice have been found to be highly convenient for the study of bacterial pathogenicity. However, mice have shown several drawbacks in *C. jejuni*-infection including sporadic and non-long-lasting colonisation as well as the absence of clinical manifestations of disease (Bereswill et al. 2011). This is partly due to that the commensal microbiota of conventional mice confers resistance against *C. jejuni* colonisation (Bereswill et al. 2011). To circumvent this problem, an optimised murine model has been developed by means of eradicating the murine gut microflora with antibiotic-cocktail treatment (Heimesaat et al. 2006). This secondary abiotic murine model was shown to exhibit a physiologically developed immune system and possesses susceptibility to *C. jejuni* infection, which renders this model suited for examination of the long-term evolution and host adaption of *C. jejuni in vivo* (Bereswill et al. 2011). In addition, given that interleukin (IL)-10 as an anti-inflammatory mediator is engaged in limiting *C. jejuni*-induced immunopathology in host and lack of the IL-10 was proven to have a profound impact on increasing the susceptibility of mice to *C. jejuni* induced enteritis, the IL-10 deficient mice were

developed and used in standardised and reproducible analyse of *C. jejuni* colonisation and immunopathology (Mansfield et al. 2008; Bereswill et al. 2011). This murine model represents a powerful study tool that has been well-documented from many studies (see more in (Heimesaat et al. 2017; Schmidt et al. 2019; Haag et al. 2012; Mousavi et al. 2020) and was used for the investigations *in vivo* in the current study.

In contrast to the scarcity of animal *in vivo* model, *in vitro* cell infection models for *C. jejuni* are highly diverse. *C. jejuni* has great permissiveness to interact with a wide range of different eukaryotic cell types (Backert and Hofreuter 2013). It has been reported that the internalisation efficiency of *C. jejuni* is strongly cell-line dependent. Thereby, the choice of cell line has to be depended on the specific experimental purpose (Backert and Hofreuter 2013). Overall, *C. jejuni* favours to invade epithelial cell lines of human origin as opposed to invade the cell lines of non-human origin (Backert and Hofreuter 2013). The most common used cell lines are the human intestinal cell lines Caco-2, T84 and INT-407 or HT-29. Among them, polarised cell model has highly differentiated mucosal epithelium and strong structural integrity, providing an environment that is closer to the *in vivo* conditions present in human gut (Konkel et al. 1992). Hence, a polarised cell model is more suitable for analysing the microbial effects on the permeability of host cells, transmigration mode and cell invasion (Backert and Hofreuter 2013). Since *C. jejuni* is a typical mucosal pathogen and mucus colonisation is a predominant step during infection, a cell line that produces or secrets mucin would be more effective to study adherence and invasion of *C. jejuni* (Backert and Hofreuter 2013). The cell line used in the current study is a sub clone of HT-29 cell line (HT-29/B6) which is polarised and has been shown to produce a mucus layer (Kreusel et al. 1991) providing an appropriate model for *C. jejuni* interaction.

2.3. Virulence factors of *Campylobacter jejuni* pathogenesis

2.3.1. Motility

C. jejuni has unusual motility, especially in viscous substances, which is attributed to the presence of the single flagellum at one or both ends of the bacteria and the helical shape of cell (Bolton 2015). Motility system in *C. jejuni* consists of flagella and chemosensory system, both are essential for colonisation of intestine. The chemosensory system drives flagella movement as the environmental condition varies, aiding in bacterial survival in various ecological milieu encountered in the GI tract (Bolton 2015). As a virulence factor for *C. jejuni* infection process, the importance of motility is best documented by the inability of flagellar mutants to colonise the intestine of experimental animal *in vivo* (Nachamkin, Yang, and Stern 1993).

The polar flagella of *C. jejuni* are secreted by the flagella type-III secretion system (T3SS) and polymerised at the distal (Cullen and Trent 2010). The flagella of *C. jejuni* is comprised of rod, hook and protofilament consisting of FlaA and FlaB subunits (Cullen and Trent 2010). Compared with other bacterial, the highly *O*-glycosylated flagellin and the absence of Toll-like receptor (TLR)5 (conserved regions of bacterial flagellin) are two unusual structural features of *C. jejuni* flagella (Guerry 2007). In addition to the key role of flagella in motility, these two features have been studied to be essential for *C. jejuni* colonisation of mucous lining in GI tract previously (Yao et al. 1994). Moreover, flagella are also vital for *C. jejuni* invasion to epithelial cells, as mutation in flagella resulted in a dramatically reduced internalisation of *C. jejuni* into host cells *in vitro* (Bolton 2015; Dasti et al. 2010; van Vliet and Ketley 2001). Gene *flaA* encodes the major flagellin protein FlaA which is an important component of extracellular filament of *C. jejuni*. Mutation of *flaA* gene can significantly reduce motility, thereby reducing adhesion and invasion capability of *C. jejuni* during host infection (Bolton 2015; van Vliet and Ketley 2001; Young, Davis, and Dirita 2007). In addition, flagella are further used as secretion system to secrete non-flagellar proteins that are potentially associated with the virulence phenomenon. The analysis of genome sequence of strain *C. jejuni* NCTN 11168 suggested that more than 50 genes are predicted to be involved in the assembly of the flagella (Parkhill et al. 2000). However, the exact mechanism underlying the regulation of the flagella genes in *C. jejuni* remains to be elucidated.

During colonisation, the primary chemoattractant for *C. jejuni* is the mucins and glycoproteins encountered in GI tract (Alemka, Corcionivoschi, and Bourke 2012). *C. jejuni* is also attracted to the chemoattractants like metabolism substances of L-aspartate and L-cysteine. Instead, *C. jejuni* is strongly repelled by bile components (Bolton 2015). Such movement is oriented by the mechanism of chemotaxis, by which bacteria sense and subsequently move towards favourable conditions (Bolton 2015). Like many other bacteria including *Salmonella typhimurium* (*S. typhimurium*), *Escherichia coli* (*E. coli*)*, C. jejuni* invades the host and locates the primary colonisation sites such as the mucus filled crypts in colon and ceca (Bolton 2015). *C. jejuni* shows chemotaxis to mucins and can bind to as well as reproduce in both human and chicken mucus (Alemka, Corcionivoschi, and Bourke 2012). Interestingly, *C. jejuni* can densely colonise the mucus layer of chicken, but without adhere to or invade in epithelial cells or even cause any disease in chicken (Alemka, Corcionivoschi, and Bourke 2012). The difference between the pathogenicity of *C. jejuni* in human and the commensalism in chicken or other poultries can be partly due to the difference in composition of mucus across species and/or the modulation of mucins (Alemka, Corcionivoschi, and Bourke 2012). These differences might further influence the chemotaxis and motility of *C. jejuni* and consequently impact on

colonisation and penetration of the mucus layer (Alemka, Corcionivoschi, and Bourke 2012). Herein, chemotaxis is a prominent virulence determinant of *C. jejuni* in the colonisation (Bolton 2015). For instance, chemotaxis protein Y encoded by gene *cheY* is known to interact with the flagellar motor causing clockwise rotation. The complete mutation in gene *cheY* resulted in a non-chemotactic but motile phenotype of *C. jejuni*. Yao et al. have reported that this *cheY* mutant possessed an increased capability of adhesion and invasion of INT-407 cells but inability of colonisation in mice (Yao, Burr, and Guerry 1997). Moreover, mutation in genes encoding methyl-accepting chemotaxis protein also resulted in a severe reduction of *C. jejuni* colonisation in chicken (Hendrixson and DiRita 2004).

2.3.2. Adhesion and invasion

In the course of colonisation, *C. jejuni* can cross the mucus layer and subsequently adhere to and invade in the epithelial cells followed by producing one or more cytotoxins (Bouwman et al. 2014). Hence, adhesion and invasion are recognised as important features in *C. jejuni* pathogenesis (Javed et al. 2010; Aguilar et al. 2014).It has been reported that the adherence capability of *C. jejuni* closely affects severity of clinical symptoms in infected individuals (Fauchere et al. 1986). The precise molecular mechanism triggering adhesion of *C. jejuni* to eukaryotic epithelial cells is revealed as a multifactorial process, yet not fully understood (Backert et al. 2013). A variety of bacterial gene have been identified to be potentially mediating the interaction of *C. jejuni* with different host cell lines. These genes are involved in encoding a wide range of factors including flagellar apparatus (*pseA*), lipooligosaccharide (LOS) biosynthesis (*galE*), sialyltransferase (cst-II gene), *N*-glycosylation (*pglB*) and fibronectin-binding proteins (*cadF*, *flpA*) (Backert et al. 2013). In particular, the outer membrane protein CadF is involved in the synthesis of a fibronectin-binding outer membrane protein promoting attachment to glycoproteins of epithelial cells (Bolton 2015; Rubinchik, Seddon, and Karlyshev 2012). Fibronectin-like protein (FlpA) binds specifically to fibronectin in extracellular matrix facilitating the host cell adhesion and colonisation (Flanagan et al. 2009). Both CadF and FlpA are required for the delivery of Cia effector proteins, which initiates the internalisation of *C. jejuni* (Bolton 2015; Kreling et al. 2020). Application of mutagenesis-based experiment promoted our understanding of several other genes involved in adhesion and invasion of host intestinal epithelial cells (IEC). For example, the *Campylobacter* adhesion protein A (CapA) is a surface-exposed lipoprotein that has implications in adherence and penetration of human epithelial cells and colonisation of chicken gut (Kreling et al. 2020; Tareen et al. 2013). Similar to the CapA, Cj0091 is another lipoprotein that has been shown to mediate adherence of *C. jejuni* to INT-407 cells and contribute to the adhesion and colonisation in chicken *in vivo* (Oakland et al. 2011).

On the other hand, it seems that some adhesion factors of *C. jejuni* function differently in the interaction with different host cells. For instance, surface-exposed lipoprotein JlpA is described to be necessary for an efficient adherence of *C. jejuni* to HEp-2 epithelial cells and the activation of NF-κB and p38 MAP kinase (Rubinchik, Seddon, and Karlyshev 2012; Tareen et al. 2013). However, the findings from another group on the role of JlpA in cellular adherence of *C. jejuni* are not consistent. The mutation in *jlpA* gene was shown to barely affect the ability of *C. jejuni* to adhere to chicken epithelial cells and human T84 cells (Rubinchik, Seddon, and Karlyshev 2012). Additionally, PEB1, a periplasmic component of an aspartate/glutamate ABC-transporter, plays a mediatory role in adhesion and invasion of human epithelial cells (Hela cells) and is predominant in the intestinal colonisation of mice (Tareen et al. 2013). However, there are other studies showing opposite that the *peb1* mutant barely exhibited difference in adhesion to T84 cells as well as chicken epithelial cells compared to the wild type (Flanagan et al. 2009; Novik, Hofreuter, and Galan 2010).

Upon infection *C. jejuni* firstly adheres to epithelial cells, from which a subpopulation invades the host cells. Thus, the binding to cells of *C. jejuni* is prerequisite for host cell invasion (Silva et al. 2011). Although adherence and/or invasion capabilities among *C. jejuni* isolates differ considerably, a positive correlation between properties of adherence and invasion of *C. jejuni* strain seems to be exist (Backert and Hofreuter 2013). However, an effective invasion process is not solely dependent on the efficient adhesion. Many *C. jejuni* proteins, oligo- and polysaccharide structures have been shown to play a pivotal role in the process of invasion. It has been reported that microtubules and the actin-cytoskeleton are involved in *C. jejuni* invasion (Alemka, Corcionivoschi, and Bourke 2012). Previous studies demonstrated that *C. jejuni* invasion of INT407 cells can be blocked by depolymerisation of microtubules (Hu and Kopecko 1999). LOS and capsule of *C. jejuni* due to their structure properties also participate in the adherence and invasion process and endow an influence on the outcome of infections (Backert and Hofreuter 2013). However, it still remains to be elucidated whether the contribution of these carbohydrates to the cellular invasion is owing to adherence or the entry process. Furthermore, certain *C. jejuni* genes have been identified using mutational study and gentamicin protection assay (GPA) (Cróinín and Backert 2012), for instance, the *Campylobacter* invasion antigens (Cia) and *iamA* encoded invasion-associated protein. To cope with the host environment, *C. jejuni* can alter its gene expression profile and thereby produce Cia. The Cia proteins are exported by the bacterium's T3SS and delivered to host cells, consequently promoting maximum cell invasion and intracellular survival (Buelow et al. 2011; Konkel et al. 1999). Previous study found that the mutagenesis of gene *ciaB* resulted in a significant reduction of internalisation of *C. jejuni* into cultured cells (Konkel et al. 1999; Rivera-Amill et al. 2001). However, this result could not be confirmed by other studies. Work

from Novik et al. illustrated that *ciaB*-deficient mutant of *C. jejuni* 81-176 has no measurable defect in entering cultured T84 cells (Novik, Hofreuter, and Galán 2010). The cellular mode of *C. jejuni* invasion seems to depend on the interplay of particular bacterial strain and host cells. Overall, there is still lack of direct evidence that any identified gene products can induce invasion directly (Cróinín and Backert 2012). Of note, the efficiency of interaction of *C. jejuni* with host cells is strongly dependent on the specific properties of bacterial strain and the cultured epithelial cell line, which consequently confers the variety on the outcomes of different *in vitro* infection studies. Therefore, the characterisation of genes related to adherence and invasion of *C. jejuni* varied significantly in different studies (Backert and Hofreuter 2013), which greatly increases the difficulty of understanding the pathogenicity of *C. jejuni*.

Many enteropathogens use “zipper” or “trigger” invasion mechanism to initiate their own uptake into target eukaryotic cells to subvert the host cell signalling pathways (Cróinín and Backert 2012). The “zipper” mechanism is induced by bacterial surface proteins consisting of adhesins and invasins that bind to host cell receptors followed by internalisation (Fig. 4A). By the “trigger” mechanism, T3SS and type-IV secretion system (T4SS) inject bacterial proteins to specifically mimic or hijack host cell factors and thus trigger the bacterial uptake process (Fig. 4B) (Cróinín and Backert 2012). However, compared with other enteropathogens, the knowledge concerning the *C. jejuni* invasion process lags largely behind, which could be partly due to the lack of the typical bacterial adhesins, toxins or classical T3SS and T4SS (Cróinín and Backert 2012).

To establish a successful infection, besides adherence to the enterocytes and invasion into the epithelial cells, intracellular survival as well as transmigration across the intestinal epithelium are equally indispensable processes during *C. jejuni* infection (Backert and Hofreuter 2013). By far most well-known transmigration mechanisms are the transcellular and the paracellular pathway. The transcellular route is described by crossing the epithelial barrier via attaching and invading at the apical side and escaping at the basal aspect of the cells. Through the paracellular route, *C. jejuni* crosses epithelial layer by breaking the tight and adherence junction complexes between neighbouring cells and subsequently enters at the basal side of intestinal cells or through the lamina propria (Backert and Hofreuter 2013). How *C. jejuni* travel across IEC, either through transcellular or paracellular pathway or by both pathways, is still not completely deciphered and hence requires more studies to unravel.

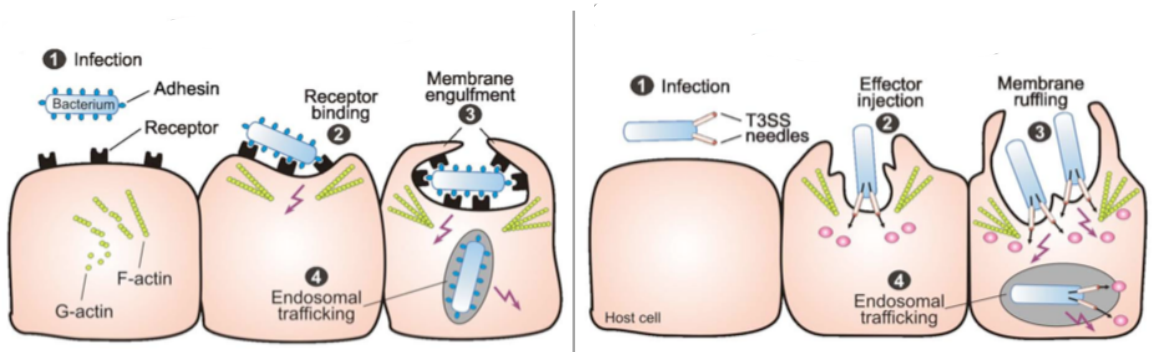

Figure 4. Mechanisms of bacterial invasion into non-phagocytic host cells. This figure was reproduced from reference (Cróinín and Backert 2012).

2.3.3. Intracellular survival

Survival mechanisms of *C. jejuni* in eukaryotic cells are still not fully understood. The intracellular fate of *C. jejuni* is likely similar to other intracellular pathogens that are capable of surviving and replicating within hostile environment in intestinal tract. Nevertheless, *C. jejuni* might have developed specific adaptation mechanism to survive within the host cells (Watson and Galan 2008). Watson et al. previously found that *C. jejuni* was co-localised in a vacuole-like compartment, which was separated clearly from the lysosomes within the host cytoplasm (Watson and Galan 2008). This specific compartment, now known as the *Campylobacter*-containing vacuole (CCV), is found to avoid fusing with lysosomes and clustering around nucleus. Once inside the IEC, *C. jejuni* exists within this unique compartment by deviating from the classic endocytic pathway (Watson and Galan 2008). This might indicate that *C. jejuni* has evolved specific adaptive strategy to traffic within host cells and to avoid from fusing with lysosomes, consequently facilitating the colonisation (Watson and Galan 2008). However, the precise mechanism by which *C. jejuni* manipulates the internal environment of the CCV to deviate from the typical endocytic pathway remains to be demonstrated. The genes related to *C. jejuni* intracellular survival and translocation are also largely unknown. Work from Buelow et al. observed that the mutation in gene *cial* caused a severe reduced intracellular survival ability of *C. jejuni* within INT-407 cells (Buelow et al. 2011). Invasion antigen CiaI was found to be involved in efficient intracellular survival of *C. jejuni*, since CiaI plays a role in preventing the delivery of the CCV to lysosomes and modifying the internal environment of the CCV (Buelow et al. 2011). Nevertheless, more insights are required to unravel the molecular basis of intracellular survival of *C. jejuni*.

2.3.4. Further factors important for colonisation

2.3.4.1. Toxin

Numerous cytotoxins produced by *C. jejuni* are engaged in the infection of host cell. Thus far, only cytolethal distending toxin (CDT) is well characterised and considered as a prominent potential virulence factor. It functions as immune modulator and plays a vital role at invasiveness (Biswas et al. 2006; John et al. 2017). CDT consists of three subunits (CdtA, CdtB and CdtC) that confer the functional activity to the toxin. Toxin protein CdtB is in charge of the activity and toxicity of CDT, while binding proteins CdtA and CdtC are responsible for binding toxins to cell membrane (Bolton 2015). The CDT toxin from *C. jejuni* was shown to cause cell cycle arrest in growth pre-mitotic phase (G_2/M) in epithelial cells (Whitehouse et al. 1998). Moreover, CDT is able to induce cell distension and elongation, swelling and eventually progress into cell death in various cell lines (Bolton 2015; Dasti et al. 2010).

2.3.4.2. Lipooligosaccharide

LOS is an integral component of *C. jejuni* cell membrane consisting of a non-repeating oligosaccharide (with a conserved inner core and a variable outer core region) and a hydrophobic lipid A portion (Hameed et al. 2020). The gene encoding proteins involved in biosynthesis of LOS core is highly variable, leading to a great diversity of cell surface LOS structure in *C. jejuni* (Hameed et al. 2020). LOS molecules has been recognised as a major virulence factor of *C. jejuni* that can serve as a protective barrier to evade immune system of the host (Lee et al. 2011; Backert and Hofreuter 2013). More specifically, *C. jejuni* can synthesise sialic acid residues endogenously. This glycan portion can mimic host molecules making the terminal structures identical to host glycan structures. This mimicry effectively mediates host-pathogen interaction and contributes to bacterial pathogenesis (Poole et al. 2018; Brockhausen 2014). Ganglioside-mimicking LOS epitopes in *C. jejuni* has been proved to be directly involved in this molecular mimicry, which has implication for the induction of anti-ganglioside antibody (Brockhausen 2014; Perera et al. 2007). Overall, the LOS differs *C. jejuni* from other enteric pathogens owing to the capability of *C. jejuni* to mimic the carbohydrate moieties of human gangliosides present on peripheral nerves. This molecular mimicry may result in a cross-reaction of produced anti-ganglioside with host ganglioside rather with *C. jejuni* LOS structures, which can give rise to autoimmune disease such as GBS (Same and Tamma 2018; Poole et al. 2018; Brockhausen 2014; Backert and Hofreuter 2013).

2.3.4.3. Glycosylation pathways

O-linked glycosylation and *N*-linked glycosylation are two glycosylation systems possessed by *C. jejuni*. Both systems are thought to play a critical role in the interaction of bacteria with the

host and the environment (Burnham and Hendrixson 2018). *O*-linked glycosylation was reported to be essential for modification and assembly of flagellar filament, thereby playing an important role on motility and flagella-dependent adherence (Karlyshev, Ketley, and Wren 2005; Day, Semchenko, and Korolik 2012). LOS and capsular polysaccharide (CPS) are immunogenic surface *O*-linked glycans expressed by *C. jejuni*. These *O*-linked glycans have a variety of glycosylation structures and have important implications in immunity of *C. jejuni* infection (Day, Semchenko, and Korolik 2012). Moreover, the side chain on LOS (formed by short *O*-linked glycans) helps to reduce non-specific binding to the mucin glycoproteins, thereby promoting *C. jejuni* to penetrate the mucus barrier and to successfully colonise epithelial cells (Young, Davis, and Dirita 2007). In contrast to the high diversity in the expression of the *O*-linked glycans produced by different strains, the *N*-linked glycans structure produced by *C. jejuni* is highly conserved in all strains (Day, Semchenko, and Korolik 2012). Moreover, *N*-linked glycosylation can modify a wide range of periplasmic and outer membrane proteins with specific glycans. The modification on these proteins are suggested to affect adherence, invasion, colonisation in mice and immunogenicity of *C. jejuni* infection (Burnham and Hendrixson 2018; Day, Semchenko, and Korolik 2012).

2.4. Immune response to *Campylobacter jejuni* infection

The human GI tract has a complex immune system that consists of cells, tissues, and immune effector molecules. They constantly and efficiently communicate with each other to eliminate invading microbial pathogens (Masanta et al. 2013). In addition to providing the host defence, IEC lining the GI tract are very important in microbial sensing and producing specific immune responses. IEC express a variety of pattern recognition receptors (PRR) composed of TLR and nucleotide oligomerization domain-like receptors (NLR). These receptors can engage with PAMPS (pathogen associated molecular patterns), which triggers cytokine production and other proinflammatory responses as well as activates adaptive immune system (Abreu 2010; Lavelle et al. 2010). Of the PRR, TLR are the most widely studied innate immune receptors. TLR have important implications in recognising specific pathogen ligands like flagella, LOS and initiating the immune defence against intruders (Lavelle et al. 2010). TLR signalling plays a crucial role in IEC proliferation, maintenance of tight junctions and antimicrobial peptide expression (Abreu 2010). TLR signaling also influences the production of trefoil factor family (TFF)3 which facilitates repair intestinal injury damage (Abreu 2010). During the host-bacterial interaction in the GI, pathogens may influence the inflammatory process by means of TLR signalling and activation of NF-*κ*B (Tang, Forsyth, and Keshavarzian 2011). The virulence factors of *C. jejuni* induce a proinflammatory response that is propagated by innate immune cells and modulated by the cells of the adaptive immune response. During *C. jejuni* infection,

adhering and invading of *C. jejuni* are detected by TLR4 and nucleotide-binding oligomerization domain (NOD) 1/CARD4, respectively. The binding of bacterial cell wall compounds with NOD or to TLR can induce innate immune responses and further activate NF-κB (Masanta et al. 2013).

Furthermore, cytokines are reported to be of importance in regulation of the intensity and duration of immune responses. Also, cytokines play a role in mediating various functions including chemotaxis, stimulation proliferation, and induction of apoptosis, among others (Al-Banna, Cyprian, and Albert 2018). The secretion of a range of cytokines can be induced by the virulence factors of *C. jejuni*. Briefly, the initiation of inflammation is predominantly controlled by IL-1β and TNF-α, and perpetuated using IL-8 and IL-6 to recruit and activate immune cells. IFN-γ, IL-17 as proinflammatory cytokines follow the recruitment and activation of T cells. IL-4 and IL-10 as anti-inflammatory cytokines are produced to activate humoral immunity as well as to control inflammation and tissue repair (Al-Banna, Cyprian, and Albert 2018). Among those cytokines, IL-8 is one of the major proinflammatory cytokines involved in both innate and adaptive immunity (Xue et al. 2014). Production of IL-8 is known to be crucial in diarrhea development and infection clearance (Aguilar et al. 2014). It has been shown that *C. jejuni* can induce IL-8 secretion via activating the TLR signalling adaptor MyD88 and NF-κB by flagellum and CDT during their colonisation, invasion and transmigration in human intestinal epithelial cells (Zheng et al. 2008). In the early phase of *C. jejuni* infection, proinflammatory cytokines like IL-8, IL-6 and TNF-α are dramatically produced to a high level promoting the development of the inflammatory cascade and the intestinal inflammation (Al-Banna, Cyprian, and Albert 2018).

2.5. Mucus layer - a frontline defence barrier

Human infection with enteropathogenic bacteria occurs via the oral route following ingestion of contaminated food and water and/or transmission through person to person (Young, Davis, and Dirita 2007). GI tract is the main infection niche of the enteropathogens. It is also harbouring microbiota and maintains a mutualistic benefiting symbiotic relationship (Johansson, Sjovall, and Hansson 2013). GI tract can form mucosal border between the external environment and internal milieu serving as the uttermost defence to prohibit harmful components from accessing the underlying tissues or entering the blood stream (Johansson, Sjovall, and Hansson 2013; Allaire et al. 2018). However, different from commensal microbes, enteropathogens are able to subvert the various protective intestinal barriers, ultimately infect and cause damage to the intestinal epithelium (Berkes et al. 2003). Moreover, infection by enteropathogens can breach the intestinal protective barriers and induce excessive stimulation

of the innate immune signalling pathways within IEC and underlying inflammatory cells, which results in enhanced production of proinflammatory cytokines and increased recruitment of immune cells to the infection site (Berkes et al. 2003; Sirard, Bayardo, and Didierlaurent 2006).

Thus, GI tract is an important site for the complex interactions between host intestinal epithelial cells, host mucus layer and enteric pathogens. GI tract has evolved an elaborate system of defence mechanism to protect host from bacteria invasion and harmful substances (Allaire et al. 2018). Among them, the mechanical barrier defence and immunological defence are the main host responses against the pathogen infection. To establish an infection, *C. jejuni* has specific traits to overcome these barriers of the GI tract and attach to IEC in the lower intestinal tract like ileum, jejunum and colon (Burnham and Hendrixson 2018; Young, Davis, and Dirita 2007; Janssen et al. 2008).

2.5.1. Structure and functional role of the mucus layer

The GI tract has the stratified mucus layer and the single layer of epithelial cells (Hansson 2012). The mucus layer overlying the IEC of GI tract is the first line where host and bacteria contact directly. The mucus layer provides a protective barrier together with glycocalyx of epithelial cells against commensals and enteropathogens accessing the underlying epithelial cells (Hansson 2012). Glycocalyx layer is a dense network underneath the mucus layer composed of highly diverse glycoproteins and glycolipids. The composition of these two layers is different. The major constituent of the mucus layer is secreted mucins while the main component of glycocalyx layer is transmembrane mucins (Hansson 2012; Linden et al. 2008). Moreover, the thin monolayer of epithelial cells form a continuous cell sheet interconnected by junctional complexes including tight junctions, adherence junctions and gap junctions or desmosomes (Hansson 2012). This study is mainly focus on the first defence line of mucus layer in colon.

The colonic mucus layer has complex structure composed of two sub-layers: the inner mucus layer which is a thinner layer of 50-100 μm thick and tightly adherent to the epithelium, whereas the outer mucus layer which is derived from and loosely attached to the inner mucus, can be up to 800 μm thick. In contrast to the inner mucus layer devoid of bacteria, the outer layer of mucus is densely colonised by commensal bacteria (Bergstrom and Xia 2013; Hansson 2012). Once impaired, the mucus barrier turns to permeable that allows bacteria to access the epithelium and inflammation ensues (Sicard et al. 2017). Previous studies have found that in the MUC2 (the gene encoding the prime mucin forming the colonic mucus layer) deficient mice, bacteria were direct in contact with epithelial cells and down to the crypts due to the lack of inner mucus layer (Johansson et al. 2008). Moreover, it has been reported that alteration in

mucosal integrity and functional defects in mucus layer are commonly associated with health problems. For instance, defects in mucin biosynthesis were suggested to be prerequires of IBD in murine models. It was found that reduction in mucus layer thickness and enhanced penetration of mucus layer by bacteria are often concomitantly occurred in ulcerative colitis (UC) patients (Bergstrom and Xia 2013). Overall, the mucus layer is dynamic and constantly renewed physical barrier that can trap enteropathogens in the outer layer and exclude the pathogens during gut movements and/or mucus turnover to protect epithelial cells. Therefore, mucus layer plays an important role in maintaining the intestinal homeostasis and avoiding inflammatory damage of the epithelial cells.

2.5.2. Colonic mucus composition: MUC2 and TFF3

The intestinal mucus consists mainly of mucins which are complex aggregation of structural glycoproteins with specific *O*-linked glycans produced by goblet cells (Sicard et al. 2017). Several mucins like MUC2, MUC5AC and MUC6 can form gel-like mucus layer, which are secreted and released by goblet cells (Johansson, Sjovall, and Hansson 2013). For colonic mucus, MUC2 is the major gel-forming mucin. It forms the skeleton of the mucus layer in colon (Johansson et al. 2011). The MUC2 mucin forms an enormous net-like polymers in mucus via: (i) assembly of MUC2 into dimeric forms linked by disulfide bonds in the endoplasmic reticulum (ER). (ii) In Golgi apparatus, MUC2 dimers are densely decorated with consecutive addition of *O*-linked glycans, turning this domain into an extended structure with a molecular mass in the range of 5 MDa. After sorting these dimers into the granules of the regulated secretory pathway, the dimers are coupled together via *N*-terminal ends by disulfide bonds into trimeric structure. This disulfide bonded di- or trimers are considered to give the MUC2 a complex net-like structure apt to form flat sheets. (iii) Once released from the goblet cells, the secreted MUC2 polymers are hydrated and expanded to form an organised flat sheet that provides the structural basis of the colonic mucus layer (Johansson et al. 2011; Hansson and Johansson 2010; Hansson 2012). As illustrated in Figure 5, the inner mucus layer is constantly renewed with the secretion of goblet cells. The inner mucus migrates upwards untill it achieves the boundary of the inner mucus layer and then transform themself into the loose mucus layer (Hansson and Johansson 2010). The inner layer is continuously renewed in a short turnover time conferring the integrity of mucus layer with a stable thickness. Although the outer layer is derived from the inner layer, its properties are completely different. The outer mucus layer is readily soluble and loose allowing the bacteria to reside or penetrate, while the inner mucus layer is insoluble, tight organised and sterile (Johansson, Sjovall, and Hansson 2013). Strikingly, it is assumed that the thickness of inner mucus layer is constant and varies from species to species. For example, the transformation of the inner to the outer mucus layer takes place at around 50 μm from the epithelia in mice, while in rats it occurs at the distance of 100

μm (Hansson and Johansson 2010). However, the mechanism of which the conversion of the tightness of mucus and how this process is controlled are still unclear.

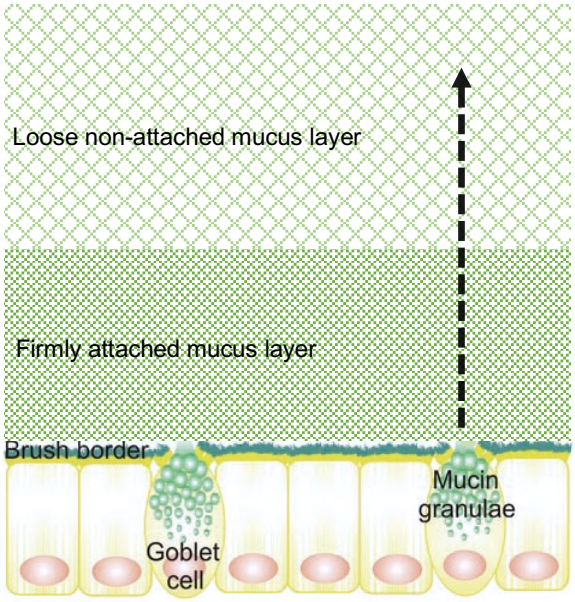

Figure 5. Structure of the mucus layers of the colon epithelium. The inner firmly attached mucus layer is continuously renewed by the goblet cells and converted to the outer loose mucus layer as shown by the dotted arrow. This figure was reproduced from reference (Hansson and Johansson 2010).

Other protective bioactive molecules in mucus include trefoil factor family peptides (TFF1, TFF2, TFF3). They are key mediators that play a role in maintaining and repairing the GI tract. TFF peptides are expressed throughout the whole GI tract and are integral constituents of mucus barrier. They are very important in regulating the viscosity of mucus layer and interacting with the proteins and receptors (Aihara, Engevik, and Montrose 2017). Herein, TTF3 is mainly present in intestine and co-secreted with MUC2 by colonic goblet cells. TFF3 is proposed to cooperate with MUC2 to ensure the elasticity and viscosity of mucin MUC2 and to enhance the structural integrity of colonic mucus barrier (Hanisch et al. 2017; Kim and Ho 2010). It has been reported that TFF3 and MUC2 working together was more effective in mucosa protection as compared with either one alone (Kindon et al. 1995). TFF3 plays a pivotal role at promoting epithelial repair and inhibiting apoptosis, thus promoting the epithelial barrier function. More specifically, TFF3 can regulate cell junctional complexes via modulating the expression of the tight junction proteins (claudin-1, claudin-2). Thus, TFF3 serves specific function in decreasing paracellular permeability and facilitating cell migration to seal the impaired area from luminal contents (Aihara, Engevik, and Montrose 2017). TFF3 also has

important implications in modulation of the resistance to IFN-γ/TNF-α-induced cell apoptosis (Hanisch et al. 2017).

2.5.3. Interaction of mucus layer and bacteria

2.5.3.1. Dynamic protection of the mucus layer against pathogens

The mucus layer overlying the IEC is the first site of the contact between the host and gut bacteria. The commensal microbiota in mucus plays a role in prevention of rapid colonisation of enteric pathogens, since it can provide colonisation resistance to enteric pathogens by competing for necessary nutrition and mucin receptors (Alemka, Corcionivoschi, and Bourke 2012). Further, commensal residence is of benefit to maintaining integrity of mucus layer. Previous *in vivo* studies demonstrated that the presence of microbiota greatly affected the thickness and composition of the mucin in a germ-free rat model (Sicard et al. 2017). Microbiotas are able to modulate the composition of mucin via mucus producing cells. Specifically, some of commensal bacteria hold capability to enhance differentiation of goblet cells and expression of mucin-related genes (Sicard et al. 2017). In addition, different probiotic agents can stimulate MUC2 production to improve the pathogen resistance of mucus. The bacterial fermentative product like butyrate is also found to be a stimulator of MUC2 production by goblet cells (Sicard et al. 2017). Moreover, immune components harboured in mucus like the antimicrobial peptides are also antagonistic towards pathogens (Alemka, Corcionivoschi, and Bourke 2012). For instance, secreted IgA is anchored in colonic outer mucus layer via combined interactions with mucus proteins and gut microbiota and thus provides the immune protection against pathogens (Rogier et al. 2014).

On the other hand, secretion and release of sufficient mucin by goblet cells is a response of host to pathological stimuli to maintain the mucus layer and to flush pathogenic bacteria away from the intestinal surface (Linden et al. 2008). Normally, the mucus layer is secreted slowly and constitutively via the movement of secretory granules within goblet cells through cytoskeleton. However, during pathogen infections, goblet cell secretion is commonly accelerated and the stored mucus in intracellular granules are rapidly discharged through exocytosis aided by the action of receptor mediated secretagogues (Specian and Oliver 1991).

Furthermore, mucin polymers can profoundly affect the bacterial behaviour and virulence. Previous data revealed that the proliferation and adhesion of *H. pylori* were strongly dependent on the gastric mucin environment (Skoog et al. 2012). Additionally, mucin glycoproteins play a role at inhibiting pathogens to access epithelial cells by binding and hampering bacteria in mucus. Several lines of evidence shown that the wide range of *O*-linked glycans on mucin proteins are used as ligands for bacterial adhesins. For instance, *C. jejuni* can interact with

mucin through the interaction of outer membrane protein with different human histo-blood group antigens (HBGAs) expressed in mucosa (Sicard et al. 2017). Moreover, to cope with pathogenic attack, qualitive changes of mucin may occur including alteration in biochemical and physical properties of mucus layer and related mucins (Deplancke and Gaskins 2001). Changes of mucus glycosylation, such as altered length of the oligosaccharide chains and varied mucus viscosity, have important implications in microbial adhesions and the capability of bacteria to degrade the mucus (Van Klinken et al. 1999; Morita et al. 1993). It has been reported that sulfation and sialylation are important vehicles to protect the intestinal mucins from bacterial degradation (Van Klinken et al. 1999).

2.5.3.2. How do bacteria cope with the mucus barrier?

Despite the complex characteristics of mucus layer and multiple levels of defence it provides, enteric pathogens are able to breach mucus barrier by employing multiple strategies. In order to destabilise mucus gel and increase the permeability of the mucus barrier, some pathogens can utilise mucinolytic enzymes including mucinases, sialidases, glycosulfatases and sialate *o*-acetylesterases, among others to degrade the specific glycoside linkage (Alemka, Corcionivoschi, and Bourke 2012). For instance, *E. coli* has been studied to secret mucinase Pic to degrade mucins aiding in intestinal colonisation (Liu et al. 2020). For *C. jejuni*, enzymically degradation of mucus could be a strategy employed by C*. jejuni* to permit colonisation. As previous study reported, several mucin-degrading enzymes (the sialoglycoprotease Cj1344c, the murein lytic glycosylase Cj0843c and the sulfoglycolases Cj0256 and Cj1055c) shown upregulation in the presence of MUC2 to help *C. jejuni* move through the mucus gel (Tu, McGuckin, and Mendz 2008). Further, chemotaxis is integral for *C. jejuni* to penetrate the mucus barrier and to enter the unique colonisation niches (Alemka, Corcionivoschi, and Bourke 2012). In addition to chemotaxis,, flagella-mediated motility is beneficial for the majority of enteropathogens to penetrate the mucus layer. Like *H. pylori* and *Vibrio cholera*, their motility aids propelling them pass through the mucus layer and access the underlying epithelium (Haiko and Westerlund-Wikstrom 2013).

The oligosaccharides of glycan provide a first attachment sites for bacteria, which allows bacteria to further access to epithelial cells (Naughton et al. 2013; Poole et al. 2018). Many pathogens use glycans presenting on the surface of host cells as the targets of bacterial toxins for attachment and the invation (Naughton et al. 2013; Poole et al. 2018). On the other hand, most pathogens are able to use strategies including modification of host glycan and immune evasion to promote colonisation (Poole et al. 2018). Upon pathogenic process, bacteria can use a range of glycosyltransferases and glycosidases to modify host glycans adding in host adaption, specifically in accessing glycans as carbon sources and changing host glycan to cell

adhesion. Also, bacteria are able to evade host immune system via expressing glycans on proteins to produce surface structures to either inhibit host immune functions or mimic host glycan structure to subvert host recognition (Poole et al. 2018; Naughton et al. 2013; Kreisman and Cobb 2012). For instance, many bacteria like *C. jejuni*, *Pasteurella multocida* have been described to encode α2, 3-sialyltransferases in their genome. This enzyme adds terminal sialic acids on microbes surface to mimic molecular structure of host, thereby facilitating colonisation and immune evasion (Kreisman and Cobb 2012).

Furthermore, pathogens can alter mucus during inflammation (Sicard et al. 2017). Factors including proinflammatory cytokines, microbial products like LPS and bacterial effectors like extracellular proteases SPATE can all cause accelerated secretion of mucin into the intestinal lumen (Deplancke and Gaskins 2001). Numerous studies have proved that MUC2 expression was stimulated by proinflammatory cytokines including IL-1, IL-6, IL-8 and TNF-α in human intestinal cell lines (Iwashita et al. 2003; Enss et al. 2000; Xue et al. 2014). *C. jejuni* was reported to be able to respond the MUC2 by modulating the expression of genes involved in colonisation and pathogenicity. Upon accessing the mucus, *C. jejuni* use MUC2 as an environmental cue to active transcriptional level of genes that encode motility proteins, cell morphology proteins and adhesins, etc., thereby promoting colonisation, adherence to and adhesion in the epithelial cells (Tu, McGuckin, and Mendz 2008). On the other hand, in response to the mucin hypersecretion of host cells, pathogens may trigger signalling pathways to retard mucin synthesis and release or to promote apoptosis of mucus secreting goblet cells. Several lines of evidence suggest goblet cells were depleted during pathogens infection of *Citrobacter rodentium*and *Shigella* as well as *Campylobacter* (Bergstrom et al. 2008).

2.5.4. Mucin glycosylation

Mucin, as integral components of mucosal barrier, are essentially *O*-glycosylated polymer proteins found in secretions and mucous membranes (Jensen, Kolarich, and Packer 2010; Struwe et al. 2015). Intestinal mucins are mainly characterised by their glycosylation. They contain repeating domains rich in amino acid proline, threonine and serine (PTS domains). These amino acid residues are attached with mucin type *O*-glycans (hereafter referred to as *O*-glycans) with different linkages (serine-linked or threonine-linked) that gives *O*-glycan different properties (Hansson and Johansson 2010; Johansson et al. 2011; Corfield 2018). Mucins also carry a relative minor amount of *N*-linked glycans, while the proportion of *O*-glycans present in mucin is around 50-80% (Naughton et al. 2014; Struwe et al. 2015). *O*-glycans are considered to have two major roles in MUC2 synthesis that is (i) *O*-glycans are comprised of charged residues that are involved in interaction with Ca^{2+} in the Golgi and goblet theca, which consequently facilitates tight packing and storage of MUC2; (ii) *O*-glycan

extension may play a role in blocking access to the polypeptide backbone and therefore prevent mucus degradation by bacterial protease (Kudelka et al. 2020). Additionally, the extensive *O*-glycosylation within the repeating domains confers mucins an extended and rod-like structure and viscoelastic properties as well as contributes to the formation of mucus gel defending against enzymatical and biochemical insults (Corfield 2018; Tran and Ten Hagen 2013; Jensen, Kolarich, and Packer 2010).

MUC2 as the main mucin in colon is a heavily glycosylated protein containing abundant *O*-glycans that multiplies MUC2 mass to five-times (Tran and Ten Hagen 2013). *O*-glycosylation is initiated by a big family of UDP-GalNAc: polypeptide *N*-acetyl-D-galacosaminyl-transferases (GalNAc-T) that catalyse the addition of initial sugar *N*-acetyl-D-galactosamine (GalNAc) to hydroxyl groups of serine and threonine residues via a *O*-glycosidic linkage. After this, extension of GalNAc occurs in a stepwise manner, yielding higher order of glycans and generating a series of mucin core structures (Arike, Holmen-Larsson, and Hansson 2017; Tran and Ten Hagen 2013; Corfield 2018). Although there are 8 different core structures that can be modified by the addition of sugars, only four core structures shown a widespread abundance. Among them, the most common extension is Galβ1-3GalNAc, known as core 1 glycan or T-antigen structure, which is generated via addition of galactose in a β1, 3 linkage to the extant GalNAc under the catalysation of the glycoprotein-*N*-acetylgalactosamine 3-β-galactosyltransferase 1 (T-synthase or C1GalT1) (Tran and Ten Hagen 2013). The core 3 structure is catalysed by β1, 3-*N*-acetylglucosaminetransferase 6 (β3Gn-T6), which adds *N*-acetylglucosamine (GlcNAc) residue in a β1, 3 linkage to GalNAc (Tran and Ten Hagen 2013). Core 1 and 3 structures can be further modified to form core 2 and core 4 structures, respectively (Tran and Ten Hagen 2013). All these core structures can be further modified or extended to create extended linear or branched structures with the involvement of a wide range of glycosyltransferases, fucosyltransferases, sialyltransferases and sulfotransferases (Tran and Ten Hagen 2013; Arike, Holmen-Larsson, and Hansson 2017).

2.5.5. Mucin type *O*-glycan biosynthesis

Most colonic *O*-glycans are mainly based on core 2 substructures formed by adding a GlcNAc to core 1 with the catalysation of β1, 6-*N*-acetylglucosaminyltransferase (C2GnT or GCNT) (Van den Steen et al. 1998; Tran and Ten Hagen 2013). In striking contrast, glycans in core 1 structure are normally small glycans that are easily terminated with sialic acid, while core 2 glycans are prone to be elaborated into longer and larger glycans (Jensen, Kolarich, and Packer 2010). The way of *O*-glycan synthesis defines the final structure of *O*-glycan. Core 1 can act as a substrate for GCNT providing GlcNAc in β1, 6 linkage to GalNAc to form core 2. Meanwhile, core 1 can also be the substrate for ST3 β-Galactoside α2,3-Sialyltransferase

(ST3GAL family), adding sialic acid to galactose in α2, 3 linkage to terminate the chain growth (Brockhausen 2014; Dalziel et al. 2001). Recent advances in glycomics analysis have revealed that cores 1 and 2 glycans are the most common mucin glycans found in mouse, whereas human colon contains primarily core 3 based and core 4 based structures (Holmen Larsson et al. 2013; Kudelka et al. 2020). Nevertheless, the terminal, such as sialic acid, sulfate, fucose and Cad, expressed in both mouse and humans are similar (Holmen Larsson et al. 2013). Thus, mouse is an ideal model for studying regulation of epithelial glycosylation (Kudelka et al. 2020). In addition, most mouse colonic *O*-glycans are mainly based on core 2 substructures formed by GCNT, of which the isoforms GCNT1 and GCNT2 are extensively expressed in mouse colon (Holmen Larsson et al. 2013).

The biosynthetic pathway of *O*-glycans is very complicated and highly diverse, owing to a narrow acceptor specificity and a diverse substrate specificity of enzymes involved in the synthesis (El-Battari et al. 2003; Le et al. 2019). Sialylation enzymes are divided in four subfamilies in accord with the specificity of their substrates and the positions of sialyl linkages in produced products, i.e., ST3GAL, β-galactosidase α2,6 sialyltransferase (ST6GAL), ST6 *N*-acetyl-galactosaminide α2,6-sialyltransferase (ST6GALNAc) and ST8 α-*N*-acetyl-neuraminide α2,8-sialyltransferase (ST8Sia) (Duan et al. 2005). ST3GAL can transfer the sialyl group from CMP-*N*-acetylneuraminate to the galactosyl residue of glycans in an α2, 3-linkage (Duan et al. 2005). There are five subtypes of ST3GAL, of which ST3GAL1 is known to be responsible for cell-cell interaction, as it can divert the *O*-linked oligosaccharide synthesis away from the polylactosamine extension (Duan et al. 2005; Prati et al. 2000). Polylactosamine is the precursor of Sialyl Lewis X (sLe^x) formation and the sLe^x is one of the mucin-type carbohydrate ligands that has the ability to inhibit inflammation via reducing the interaction between cells (Prati et al. 2000). Moreover, ST3GAL2 is known for the importance in the biosynthesis of gangliosides. It preferentially transfers sialic acid from CMP-*N*-acetylneuraminate (CMP-NeuAc) to the Galβ1-3GalNAc terminus of gangliosides (Gomes et al. 2012; Lopez et al. 2017). As one of sialylated glycans, gangliosides exist in high concentration in the peripheral nervous system and also present on the epithelial cells of the entire gut, stomach throughout to colon. Gangliosides play a role in regulation of cell function and mediation of intracellular recognition (Lopez et al. 2017; Day, Semchenko, and Korolik 2012). In terms of polylactosamine, core 2 GCNT is a main enzyme for the synthesis of this sLe^x precursor (Prati et al. 2000). GCNT is a family of glycosyltransferases that can catalyse the *O*-glycans in core 2 and core 4 structures by transferring the GlcNAc group in a branching GlcNAc β1, 6 GalNAc-linkage from UDP-GlcNAc to the GalNAc residue in precursor of core 2 or of core 4. GCNT is divided in three subtypes, of which GCNT1 and GCNT3 are only responsible for synthesis of core 2, whereas GCNT2 can synthesize both core 2 and core 4 (Prati et al. 2000; Gupta et al. 2020; Dalziel et

al. 2001). Even more intriguingly, it has been demonstrated that these two enzymes, ST3GAL1 and GCNT1, are in the relationship of competing substrates, since they use one common substrate (Galβ 1–3GalNAcα) in the *O*-glycosylation pathways in the mammal cells (Prati et al. 2000; Duan et al. 2005). In particular, ST3GAL1 can truncates *O*-glycosylation by capping Tn-antigen with sialic acid, thereby contributing to an inhibition in the formation of core 2 structure (Dalziel et al. 2001; Gupta et al. 2020). Nevertheless, it is elusive to identify the glycans and their corresponding biosynthetic enzymes involved in specific biological events. The reasons are multifarious, as the highly complex structure of glycans, variety of attachment sites of glycans and multiplicity of potentially redundant enzymes all increase the difficulty of the identification (Kurcon et al. 2015).

2.5.6. Mucin glycosylation and diseases

Last decade has been seen major advances in understanding how infection and inflammation result in host glycosylation changes, and in turn, how host glycosylation alterations cause susceptibility to infection and inflammation, as well as the significance of glycosylation on molecular and cellular function within immune system. Dysregulation in glycosylation have been implicated in certain processes as varied as inflammatory responses, autoimmunity, infection and cancer (Kreisman and Cobb 2012). Previous studies have observed altered degree of sulfation, sialylation and varied rate of glycosylation as well as changed length and complexity of mucin glycans in IBD patients. In normal human gut, mucins are sialylated and *O*-acetylated, which confers mucus resistance of degradation. However, in IBD patients, the insufficiently *O*-acetylation of sialylated mucins resulted in an increased proteolytic degradation, which impairs the viscosity and thickness of mucus (Kudelka et al. 2020).

Mucin *O*-glycosylation has been shown to be dynamic and glycan patterns are commonly altered as a secondary effect to inflammation. During intestinal inflammatory, reduced sulfation and increased sialylation of mucus as well as altered length and structure of glycan chains have also been observed, which further impacted adhesion of bacteria and the degradation of mucus by pathogens (Kudelka et al. 2020). Moreover, mucins shown some specific modification of glycosylation during malignant transformation (Byrd and Bresalier 2004). For instance, core 3 and core 4 structures are found to be reduced and the expression of C1GalT1 is increased, which promotes production of Tn-antigen associated with cancer metastasis. During malignant transformation, the sialylation of Tn-antigen is enhanced to facilitate producing truncated *O*-glycans. Moreover, the reduction of sulfation and the increase of sialylation are also found in the malignant transformation (Byrd and Bresalier 2004). The importance of *O*-glycans in selectin-carbohydrate-mediated cell adhesion events are well documented within the immune system. It is evident that inflammatory mediator TNF-α play a

promotive role in accumulating selectin molecules on the surface of endothelial cells near to the site of inflammation during infection. These selectin molecules contain glycans that can bind to leukocytes in circulation, facilitating the recruitment of leukocytes to the infection sites (Kreisman and Cobb 2012). There are many glycosylation-related knockout mice have been used to demonstrate the aberration of glycome in causing infection/inflammation. For example, a reduction of neutrophil rolling on surface expressing selectins was observed in the mice deficient in GCNT1, from which deficient neutrophil homing to the inflammation sites ensued (Kreisman and Cobb 2012). Similarly, the ST3GAL family (ST3GAL1-6) that potentially engages in the synthesis of selectin ligands is shown to play a role in E-selectin binding and leukocyte homing to inflammation sites (Ellies et al. 2002).

2.5.7. Regulatory factors of mucin

Mucin (MUC2) expression is normally controlled by transcriptional regulation with involvement of many bioactive factors including microbes, microbial products, toxins and cytokines or growth factors (as mucin secretagogues) (Kim and Ho 2010). Particularly, inflammatory cytokines such as interferons play a significant role on the expression of gel-forming mucins (Linden et al. 2008; Xue et al. 2014). Mucin expression is stimulated by proinflammatory cytokines including IL-1, IL-6, IL-8 and TNF-α in different aetiologies (Iwashita et al. 2003; Enss et al. 2000; Xue et al. 2014; Bautista et al. 2009). For instance, work from Xue et al. have demonstrated that *E. coli* O157:H7 infection or TNF-α treatment resulted in upregulated IL-8 expression and enhanced MUC2 expression presumably via MAPK/ERK1/2 signalling in epithelial cells (Xue et al. 2014). In addition, MUC2 have binding sites to NF-κB in the promoter. LPS from enteropathogens can active NF-κB mediated by Ras-mitogen-activated protein kinas (MAPK) pathway and thereby can increase MUC2 transcription in colon epithelial cells (Kim and Ho 2010). Also, inflammatory cytokine TNF-α can upregulate MUC2 transcription by means of activation of NF-κB mediated by PI3K/Akt pathways. Interestingly, instead of promoting MUC2 transcription, TNF-α can inhibit MUC2 expression via activation of JNK pathway (Kim and Ho 2010). In addition, the bioactive factor SPDEF (Sam pointed domain containing Ets) is characterised to be a major regulator of MUC2 that can promote the differentiation of progenitor cells into goblet cell and Paneth cell (Kim and Ho 2010).

TFF3, as the co-secreted protein with MUC2 by goblet cells, can behave as signalling molecules modulating immune responses, like regulating cytokine expression and immunocyte migration. Previous *in vitro* study demonstrated that TFF3 induced downregulation of proinflammatory cytokines like IL-6, IL-8 by triggering a signal transduction pathway, and enhanced the expression of antimicrobial peptides (hBD2, hBD4) in HT-29 cell line (Barrera, Sanchez, and Gonzalez 2012). On the other hand, proinflammatory cytokines in turn affect the

expression of TFF3. IL-1β or IL-6 was reported to suppress the promoter activity of TFF3 by activation of NF-κB or C/EBPβ in HT-29 cells, respectively (Dossinger et al. 2002). These may reflect the situation *in vivo* upon *Helicobacter pylori* (*H. pylori*) infection. Specifically, the inflammation or mucus metaplasia is normally accompanied with the alteration in TFFs during *H. pylori* infection. The process of *H. pylori*-induced inflammation commonly involves cytokines and activation of NF-κB, etc., of which stimulation or processes greatly influence the increase/decrease of TFFs expression (Aihara, Engevik, and Montrose 2017).

Besides, microRNAs (miRNAs) are reported to be highly participated and play a critical role in mucin regulation. MiRNAs are single-stranded small Non-coding RNA (ncRNA) molecules containing in average 22 nucleotides in length, which normally binds to the 3′ untranslated regions (UTR) of target Messenger RNAs (mRNAs) and negatively regulates gene expression at a posttranscriptional level. Due to the imperfect base-pairing with complementary sequences within mRNA molecules, the target mRNA is consequently degraded or their translation is suppressed (Gebert and MacRae 2019; Bertoli, Cava, and Castiglioni 2015). Simply based on the fact that a single miRNA can target different genes and a single target gene can be coordinately regulated by different miRNAs, therefore, miRNAs combine associated genes into a complex regulatory network concerting to control a wide variety of fundamental biological processes. They target genes controlling cellular processes including cell differentiation, proliferation and apoptosis as well as inflammation and immune response, among others. (Zhang et al. 2019; Gebert and MacRae 2019; Bertoli, Cava, and Castiglioni 2015).

There are evidence showing that miRNAs are important mediator of goblet cell development and proinflammatory cytokine expression (Liu et al. 2016; Eyking et al. 2016; Proenca et al. 2018; Eulalio, Schulte, and Vogel 2012), which are critical factors involved in mucin alteration during pathogens infection. For example, miR-125b plays a modulatory role in goblet cell differentiation and mucus secretion in asthma by repressing a specific ETS transcription factor (Liu et al. 2016). Eyking et al. using *in vitro* human Caco-2-based models shown that miR-205 signalling enhanced abundant mucin production and MUC2 expression. MiR-205 was also implicated in differentiation and enrichment of colonic goblet cells by affecting central mediators of goblet cells expansion (Eyking et al. 2016). Several mucins including MUC1, MUC4, MUC5AC as well as MUC2, among others have been reported to be modulated by miRNAs during malignant development and progression of cancer (Krishn, Batra, and Kaur 2015). For example, the alteration of MUC1 (a transmembrane protein) expression was found to be regulated by multiple miRNAs including miR-145, miR-125b, miR-1226 and miR-129 in various malignancies. Among them, miR-145 was shown to downregulate invasion and

metastasis of metastatic breast cancer cells by targeting 3'UTR of MUC1 (Krishn, Batra, and Kaur 2015). MiRNA-125b can reduce the expression of MUC1 in breast cancer cells contributing to decrease of cell growth and reduced induction of cell apoptosis and thereby functions as a tumour suppressor (Krishn, Batra, and Kaur 2015). MUC2 is the major secretory mucin in colon and its dysregulation is normally correlated with colorectal cancer development and the progression of some type of lung cancer (Krishn, Batra, and Kaur 2015). Work from Bu et al. has demonstrated that the suppression of MUC2 significantly promoted the growth rate and invasion/migration of the cells both *in vivo* and *in vitro* (Bu et al. 2011). Interestingly, miR-9 was found to be a regulatory factor of the caudal-related homeobox protein CDX2 that is known to be associated with the development of gastric carcinogenesis. MiR-9 can repress expression of CDX2 and its downstream targets like MUC2 and TFF3 by targeting 3'UTR of CDX2 leading to promotion of proliferation in gastric cancer cells (MKN45 and NUGC-3 cells) (Rotkrua et al. 2011).

Mucins are highly characterised by their glycosylation. In recent time, miRNAs have emerged as major regulators of glycome, controlling the level of glycan biosynthesis enzymes and playing a pivotal role in modulating and controlling glycosylation (Kasper, Koppolu, and Mahal 2014; Agrawal et al. 2014). Kurcon et al. has utilised the glycogen target network of miR-200-family to identify three glycosylation enzymes (ST3GAL5, ST6GALNAc5 and β1,3-glucosyltransferase B3GLCT) that control epithelial-to-mesenchymal transition in a metastatic breast cancer cell line (Kurcon et al. 2015). Moreover, recent advances in function of miRNAs have pointed out that the regulatory role of miRNAs at tumour metastasis and neuronal migration may function via modulating the glycan biosynthesis enzymes (Agrawal et al. 2014). For instance, miR-30b and miR-30d play a promotive role in metastatic growth in melanoma cells by targeting the GalNAc transferase GALNT7 (Gaziel-Sovran et al. 2011). However, whether mucin regulation is concerted by the interplay of miRNAs and glycosylation remains largely unknow.

Apart from that miRNAs play an important role in modulating mucin biosynthesis processes, it is becoming clear that miRNAs serve specific functions during microbial infections (Eulalio, Schulte, and Vogel 2012). For example, miR-125 family were shown to play an important role in immune responses to bacterial and viral infection. An up-regulated miR-125b induced by lipomannan from virulent *Mycobacterium* tuberculosis can destabilize the transcript of TNF and therefore block its biosynthesis (Sun, Lin, and Chen 2013). MiR-125a can interact with viral sequence and interfere in the viral translation, consequently down-regulating the expression of surface antigens (Sun, Lin, and Chen 2013). Moreover, miR-146 plays a role in downregulating cellular LPS sensitivity and preventing excessive inflammation. In response to

bacterial products, miR-146 was considered as a mediator involved in a negative feedback mechanism regulating TLR/ NF-κB pathway by targeting TRAF6 (TNFR-associated factor 6) and IRAK1 (IL-1R-associated kinase 1) (Staedel and Darfeuille 2013; Cortes-Marquez et al. 2018). Interestingly, Singh et al. have found that murine caecal miRNA signature may vary as the influence of endogenous microbiota and further affect the function of intestinal barrier by targeting genes related to glycosylation enzymes and junctional proteins, etc. (Singh et al. 2012). Collectively, these findings prompt the idea to discover a novel miRNA-mediated regulatory network that has implications in mucin regulation and posttranscriptional modification in the context of pathogen infection.

3. Aim and Objectives

In view of the deficiency in our knowledge on pathogenicity factors of *C. jejuni* and the molecular mechanisms of host response to *C. jejuni* infection, the identification of involved bacterial and host factors is crucial for the understanding of the molecular mechanisms triggering campylobacteriosis and the future development of effective treatment regimens. The aim of this study was to determine further *C. jejuni* pathogenicity factors and virulence genes based on a random mutagenesis approach. Moreover, another goal of this study was to identify miRNAs-mediated regulatory networks that could play a role in mucin modification and interaction by using *in vivo* and *in vitro* infection model.

The specific objectives were:

1. Identification of novel genes that may be involve in adhesion and invasion of *C. jejuni* from a constructed mutant library of *C. jejuni* NCTC 11168
2. Phenotypic characterisation of the mutated strains of *C. jejuni* with respect to adhesion, invasion, intracellular survival and host cell response
3. Determination of the host responses including mucin regulation and posttranscriptional modification in established *in vitro* and *in vivo C. jejuni* infection model
4. Identification of the potentially mucin-associated interfering miRNAs upon *C. jejuni* infection and regulatory networks concerted by miRNAs involved in mucin modification and interaction.

4. Subsuming the published work

4.1. Publication 1: *Campylobacter jejuni* genes Cj1492c and Cj1507c are involved in host cell adhesion and invasion

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Campylobacter jejuni genes Cj1492c and Cj1507c are involved in host cell adhesion and invasion

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Abstract

Background: *Campylobacter jejuni* (*C. jejuni*) has been assigned as an important food-borne pathogen for human health but many pathogenicity factors of *C. jejuni* and human host cell responses related to the infection have not yet been adequately clarified. This study aimed to determine further *C. jejuni* pathogenicity factors and virulence genes based on a random mutagenesis approach. A transposon mutant library of *C. jejuni* NCTC 11168 was constructed and the ability of individual mutants to adhere to and invade human intestinal epithelial cells was evaluated compared to the wild type. We identified two mutants of *C. jejuni* possessing altered phenotypes with transposon insertions in the genes Cj1492c and Cj1507c. Cj1492c is annotated as a two-component sensor and Cj1507c is described as a regulatory protein. However, functions of both mutated genes are not clarified so far.

Results: In comparison to the wild type, Cj::1492c and Cj::1507c showed around 70–80% relative motility and Cj::1492c had around 3-times enhanced adhesion and invasion rates whereas Cj::1507c had significantly impaired adhesive and invasive capability. Moreover, Cj::1492c had a longer lag phase and slower growth rate while Cj::1507c showed similar growth compared to the wild type. Between 5 and 24 h post infection, more than 60% of the intracellular wild type *C. jejuni* were eliminated in HT-29/B6 cells, however, significantly fewer mutants were able to survive intracellularly. Nevertheless, no difference in host cell viability and induction of the pro-inflammatory chemokine IL-8 were determined between both mutants and the wild type.

Conclusion: We conclude that genes regulated by Cj1507c have an impact on efficient adhesion, invasion and intracellular survival of *C. jejuni* in HT-29/B6 cells. Furthermore, potential signal sensing by Cj1492c seems to lead to limiting attachment and hence internalisation of *C. jejuni*. However, as the intracellular survival capacities are reduced, we suggest that signal sensing by Cj1492c impacts several processes related to pathogenicity of *C. jejuni*.

Keywords: *Campylobacter jejuni*, Mutants, Pathogenicity, Host cell response

Background

Campylobacter spp. are microaerophilic, gram-negative and motile bacteria belonging to the family of *Campylobacteraceae* [1]. *C. jejuni* is the most frequent cause of bacterial food borne disease worldwide [2–4]. The most common symptoms caused by *C. jejuni* are gastroenteritis usually accompanied by fever, vomiting, abdominal pain and bloody diarrhoea [1, 5]. In contrast to other enteropathogens, our understanding of pathogenetic mechanisms of *C. jejuni* lags behind and functions of many genes of *C. jejuni* still remain to be elucidated.

In the course of colonisation, *C. jejuni* can cross the mucosal barrier, adhere to as well as invade host epithelial cells, and produce one or more cytotoxins [3,

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6]. Extensive studies have investigated that *C. jejuni* possesses numerous pathogenicity-associated factors involved in bacterial colonisation, transmigration and intracellular survival in intestinal epithelial cells [7, 8]. Two major known transmigration mechanisms are the transcellular and the paracellular pathway [8]. The transcellular route allows *C. jejuni* to cross the epithelial layer by attaching and invading at their apical surface, followed by escaping at the basolateral membrane. The paracellular pathway is characterised by breaking the tight junction and adherens junction complexes and crossing epithelial barriers by passage between neighbouring cells [7]. It has been demonstrated recently, that the *C. jejuni* protease HtrA is involved in breaching the epithelial barrier and enables invasion of epithelial cells via the paracellular pathway [9].

Hence, adhesion and invasion are recognised as important features in *C. jejuni* pathogenesis [10, 11]. There are several adhesion and invasion related genes already characterised in many in vivo and in vitro studies. The *flaA* and *flaB* genes, encoding major flagellin proteins, are strongly associated with invasiveness of *C. jejuni* [1]. Other genes such as *cadF* have been also implicated in adhesion. It is involved in the synthesis of a fibronectin-binding outer membrane protein facilitating attachment to glycoproteins of epithelial cells [12]. Mutagenesis based experiments indicate that several other genes, for example *ciaB*, *capA* and *flgB* are involved in binding and invasion of host intestinal epithelial cells [1, 5].

Campylobacter jejuni infection can induce the expression of various cytokines [13–15]. Interleukin-8 (IL-8) is an important pro-inflammatory chemokine of intestinal epithelial cells and acts as a chemotactic factor of immune cells. It can be induced during infection by adhesion and/or invasion as well as exposure to the cytolethal-distending-toxin (CDT) produced by *C. jejuni* [13, 14, 16, 17]. While research in the past decade has focused on mechanisms of *C. jejuni* interaction with host cells, the knowledge on its intracellular fate is still limited. Only few genes have been reported to be involved in intracellular survival of *C. jejuni* [18].

Studies have proved that the intestinal mucosa behaves as the first barrier against microbial infections. Since human intestinal epithelial cells are polarised and possess a luminal mucous layer [7], adhesion, invasion and translocation ability of *C. jejuni* have been shown to depend on these characteristics. In our study, we have used the epithelial cell line HT-29/B6, a sub clone of HT-29 cell line, which has been shown to produce a mucus layer [19] providing an appropriate model for *C. jejuni* interaction.

In this study, we applied an approach utilising in vitro integration of a transposon in *C. jejuni* genomic DNA followed by natural transformation [20, 21]. A transposon mutant library was constructed in *C. jejuni* NCTC 11168, from which two mutants with altered capability to invade human intestinal epithelial cells have been selected and used for further phenotypical characterisation. The aim of the present study was to discover hitherto unknown pathogenicity factors of *C. jejuni* with respect to adhesion, invasion, intracellular survival and host cell response. We found that mutagenesis of Cj1492c and Cj1507c, two so far poorly characterised genes of *C. jejuni* NCTC 11168, has remarkable effects on adhesion and invasion of host cells in a reverse manner. However, they seem not to influence host cell pro-inflammatory responses.

Results

Transposon mutagenesis of *C. jejuni* NCTC11168

In this study, the HyperMu <KAN-1> Transposon was randomly inserted into the DNA of *C. jejuni* NCTC 11168 using a 2 h in vitro reaction catalysed by HyperMu MuA Transposase and subsequently transformed into *C. jejuni* NCTC 11168 by natural competence. This in vivo protocol successfully generated 24 kanamycin-resistant mutants in *C. jejuni* NCTC11168. Transposon insertion site was determined by sequencing with the primers included in the HyperMu <KAN> Transposon Kit and verified by gene specific PCR amplification. The sequence data proved that each mutant had a single transposon insertion and transposons were inserted randomly on *C. jejuni* chromosome (data not shown). To identify genes involved in *C. jejuni* pathogenicity, the obtained mutants were subsequently screened using invasion assays with HT-29/B6 cells. Two mutants (::Cj1492c and ::Cj1507c) that showed differing invasiveness compared to the wild type were chosen to assess detailed phenotypic studies. However, the function of mutated genes is not clarified so far. One insertion is located in the gene Cj1492c, annotated as a two-component sensor and the second one is located in the gene Cj1507c, described as a regulatory protein, involved in molybdenum and tungsten transport [22]. The maps of Cj1492c and Cj1507c genes and transposon insertion sites are shown in Fig. 1a, b. The results of a gene specific PCR are shown in Fig. 1c. Both mutants possessed an approximately 1.2 kb larger fragment compared to the parental target DNA indicating that these genes contained a transposon insertion.

Growth dynamics

Both mutants (::Cj1492c and ::Cj1507c) and the wild type were separately inoculated in Brucella broth (BB) and incubated for 48 h at 37 ℃ under microaerobic conditions and viable cell counts were determined over time. A growth curve including lag (0–8 h), exponential (8–18 h) and stationary (18–32 h) phases is presented

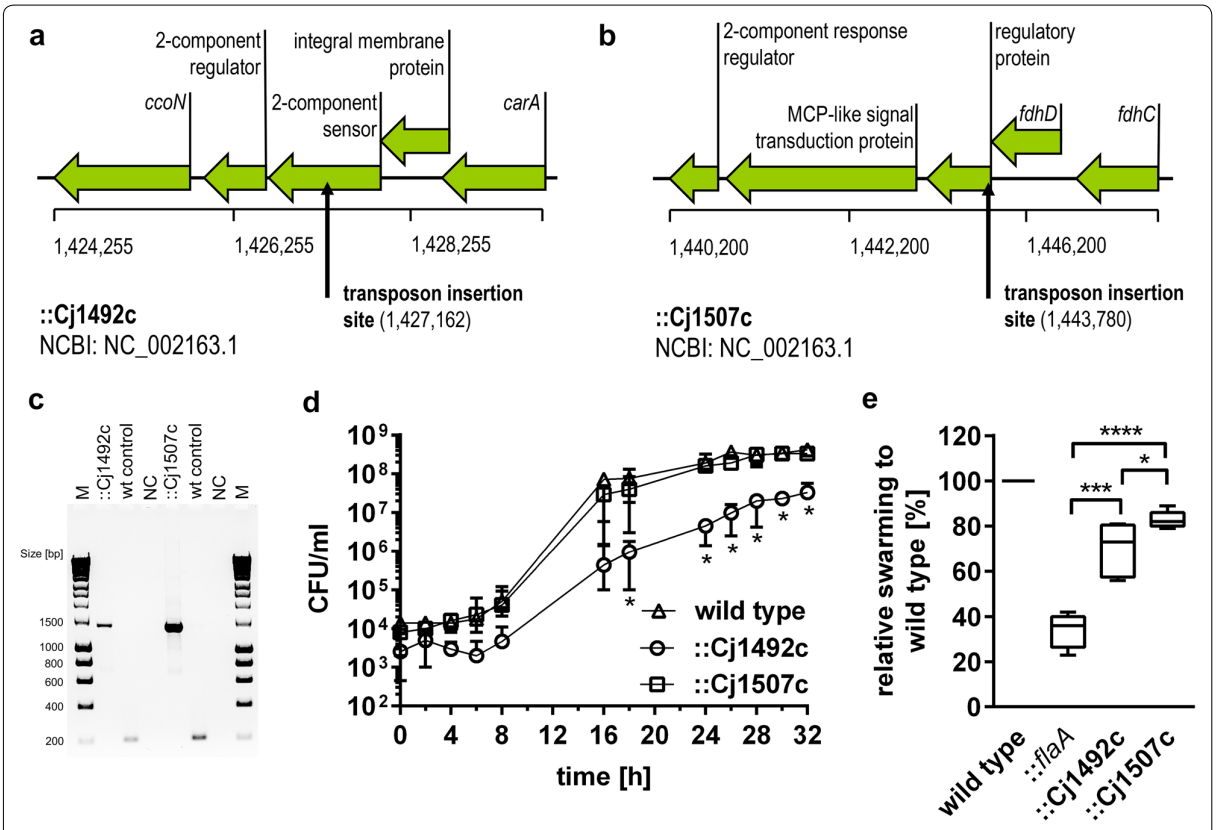

Fig. 1 Genomic locus, transposon insertion sites in *C. jejuni* NCTC 11168 mutants ::Cj1492c and ::Cj1507c and PCR verification of both mutants and phenotypic analyses of two mutants. **a**, **b** Genes are plotted as arrows in order to their genomic locations. Boxed arrows length indicates relative gene sizes. **a** The location of inserted transposon is indicated in *C. jejuni* two-component sensor gene (Cj1492c). **b** The location of the inserted transposon is indicated in *C. jejuni* regulatory protein gene (Cj1507c). **c** Agarose gel electrophoresis of genomic DNA of mutants ::Cj1492c, ::Cj1507c and wild type using gene specific primers. With integration of the transposon (1219 base pairs) the DNA fragment sizes of both mutants are around 1400 base pairs in length which are 1.2 kb larger than the target DNA of the wild type (around 200 base pairs). **d** Growth curve of wild type, ::Cj1492c and ::Cj1507c were assessed by CFU counting. Values are shown as mean value of three independent experiments. Error bars represent SD. Statistical comparison with the wild type: *$p \leq 0.05$, unpaired *t*-test. **e** Differential swarming ability of mutated strains was normalised with the wild type strain and is presented as percentage of swarming halos of wild type (100%). Columns show median value of five biological replicates and bars indicate maximum and minimum range. *$p \leq 0.05$; ***$p \leq 0.001$; ****$p \leq 0.0001$, unpaired *t*-test

in Fig. 1d. Strain ::Cj1507c grew almost identically to the wild type whereas ::Cj1492c showed apparent differences in growth dynamics. Early growth rate of ::Cj1492c was clearly retarded compared to the wild type as represented by a flatter slope of the growth curve between 6 and 18 h and approx. 100-fold lower number of viable bacteria during exponential phase. After 28 h, ::Cj1492c reached a stationary phase with significantly diminished number of viable bacteria compared to the wild type and ::Cj1507c and there was no difference between the two other strains.

Swarming ability of ::Cj1492c and ::Cj1507c

As motility is essential for host invasion [1, 18], ::Cj1492c, ::Cj1507c, wild type and a ::*flaA* mutant (used as a negative control) were tested on semisolid Mueller–Hinton agar (MH) plates to examine whether *C. jejuni* swarming ability is affected in the mutated strains. Swarming ability of the three mutants was normalised to the wild type. As expected, ::*flaA* was found to be barely motile (36%), which is consistent with previous studies [1, 23–25]. In contrast and as shown in Fig. 1e, both mutant ::Cj1492c and ::Cj1507c, showed slightly reduced swarming ability (approx. 73% and 82% of wild type swarming in MH, respectively).

Adhesion and invasion of human intestinal epithelial cells by ::Cj1492c and ::Cj1507c

Both mutants along with the wild type were tested for their abilities to adhere to and invade HT-29/B6 host

cells. Therefore, HT-29/B6 monolayers (with approx. 80–90% confluence) were infected with approx. 1×10^9 colony-forming units (CFU) of each strain. The strain ::Cj1492c showed more than threefold but statistically not significantly enhanced adhesion to HT-29/B6 cells compared to the wild type (0.037% of ::Cj1492c vs. 0.011% of wild type inoculum adhered to host cells). However, disruption of Cj1507c caused clearly and significantly ($p \leq 0.05$) decreased adhesion to host cells. Only 0.003% of ::Cj1507c inoculum adhered to HT-29/B6, which corresponds to 27% of the wild type (Fig. 2a).

In accordance with the adhesion analysis, we determined that mutant ::Cj1492c invaded epithelial cells approx. 3-times more efficiently than the wild type (0.0049% of inoculum for ::Cj1492c vs. 0.0015% for wild type, $p \leq 0.001$), while the strain ::Cj1507c showed tenfold reduced ($p \leq 0.05$) ability to invade HT-29/B6 cells compared to the wild type (Fig. 2b).

Immunodetection of intracellular *C. jejuni*

With regard to adhesion and invasion, we examined that mutation of the genes Cj1492c and Cj1507 causes contrary phenotypes. In order to determine the internalisation level of *C. jejuni* in HT-29/B6, immunofluorescence staining along with WGA and DAPI staining were performed up to 5 h post infection (p.i.). WGA is a carbohydrate-binding lectin that has high affinity for sialic acid and *N*-acetylglucosamine [26] and is used

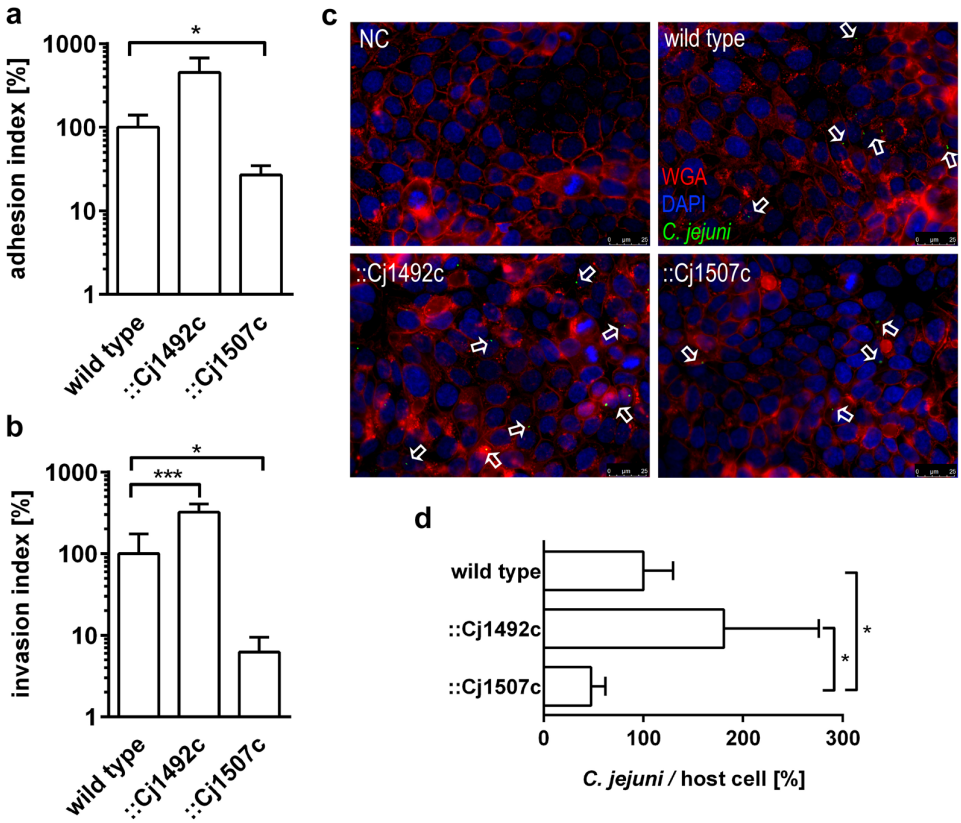

Fig. 2 Adhesion, invasion and internalisation levels of *C. jejuni* in HT-29/B6 cells and immunofluorescent detection of intracellular *C. jejuni*. Adhesion (**a**) of three tested strains to HT-29/B6 was detected at 1 h p.i.. For determination of invasion (**b**), the monolayer of HT-29/B6 was incubated with gentamicin for an additional 2 h prior to lysis. Experiments were performed at least three independent times in triplicate. The relative adhesion and invasion index were normalised with wild type and are presented as percentage of the adhesion and invasion index of wild type, respectively. Results show the mean value ± SD. *$p \leq 0.05$; ***$p \leq 0.001$, unpaired *t*-test. **c** HT-29/B6 cells and immunofluorescently labelled wild type, ::Cj1492c and ::Cj1507c (green, indicated by white arrows) and untreated control cells are shown at 5 h p.i. Cell cytoplasm membrane were stained with WGA-CF®594 (red) and nuclei with DAPI (blue). Scale bars indicates 25 µm. **d** The internalisation level of *C. jejuni* by HT-29/B6 was determined as a ratio of particles of invaded *C. jejuni* and infected cells by means of immunofluorescence microscopy and automatic particle counting using ImageJ. The relative level of internalisation was normalised with wild type and is presented as percentage of the wild type. Fluorescent particles of each strain were quantified in at least six microphotographs selected randomly for each biological replication and results are presented for four individual experiments. Asterisks show statistically significant differences between ::Cj1492c and ::Cj1507c. *$p \leq 0.05$; unpaired *t*-test

as a marker for the cytoplasm membrane of host cells. To subtract unspecific immunofluorescent signal, non-infected HT-29/B6 cells, which were stained the same as the infected samples, were used as a negative control. There was no immunofluorescent signal detected in the negative controls. As representatively shown in Fig. 2c, several bacteria seemed to be located at the intercellular spaces. No specific differences were found among the tested strains. Furthermore, we quantified the number of invaded bacteria per host cell at 5 h p.i. by means of fluorescent particle counting using ImageJ [27, 28]. The average number of wild type *C. jejuni* per host cell was ~ 0.08 bacteria/host cell. As shown in Fig. 2d, the mutant ::Cj1492c exhibited increased levels of internalisation compared to the wild type (181% of the wild type) but was not statistically significant, whereas ::Cj1507c possessed half the internalisation of the wild type (47% of the wild type, $p \leq 0.05$). These data are in line with our observations in the invasion assay shown above.

Intracellular persistence of the mutants ::Cj1492c and ::Cj1507c

We were able to show that these two mutants possess adhesion and invasion capabilities in a reverse manner but retain only slightly diminished swarming abilities compared to the wild type. Furthermore, we wanted to address whether both mutants showed altered abilities to persist intracellularly. Therefore, the number of intracellular bacteria was determined over time by examining the CFU at 5 and 24 h p.i., respectively. More than two-thirds of the intracellular wild type *C. jejuni* were eliminated between 5 and 24 h p.i. while approx. 85% of intracellular ::Cj1492c and more than 95% of the intracellular ::Cj1507c were eliminated within this time. As indicated in Fig. 3a, the mutant ::Cj1492c exhibited half the survival rate of the wild type (48.1% of wild type, $p \leq 0.05$) and the intracellular persistence of strain ::Cj1507c was only one-tenth of the wild type (9.4% of wild type, $p \leq 0.01$). Both mutants showed significantly diminished intracellular survival rates compared to the wild type. However, there was no statistically significant difference between the mutants. Regardless of the mutation, these data showed that most intracellular *C. jejuni* were killed within the initial 24 h p.i. in HT-29/B6.

Effects of mutations on host cell viability

For determining effects of *C. jejuni* strains on host cell survival, we tested the viability of HT-29/B6 cells at 5 and 24 h p.i. based on Calcein-AM/Hoechst staining [29, 30]. This assay quantitatively determines the number of viable host cells indicated by the Calcein signal versus the total counts of cells represented by Hoechst staining. To assess relative host cell survival after infection, the ratio of viable to total cell numbers was calculated in relation to non-infected cells. As shown in Fig. 3b, there was no significant difference in host cell viability among tested stains and for the duration of the experiment over 24 h.

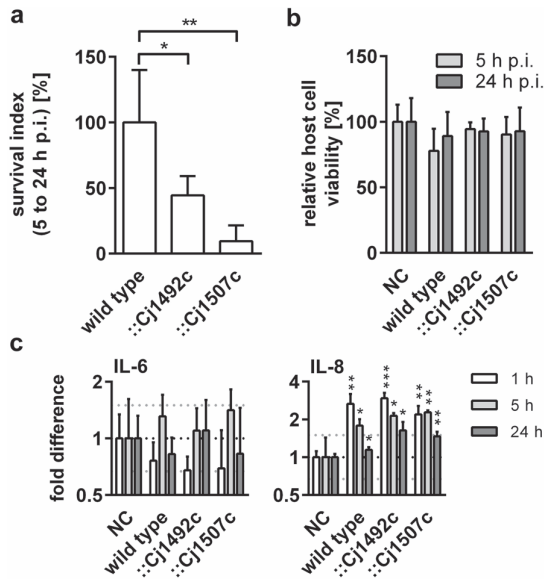

Fig. 3 Intracellular survival of *C. jejuni*, relative viability of HT-29/B6 cells and RT-qPCR analysis of IL-6 and IL-8 mRNA expression in HT-29/B6 cells after *C. jejuni* infection. **a** Intracellular survival, presented as CFU recovery rates of intracellular viable cell counts, was determined as a ratio of viable counts between 5 and 24 h p.i. The relative survival rate was normalised with the wild type and is presented as percentage of the wild type. Results represent at least six individual experiments and asterisks indicate statistically significant differences between mutants and the wild type. *$p \leq 0.05$; **$p \leq 0.01$, unpaired *t*-test. **b** Relative viability of HT-29/B6 cells after infection was measured after 5 h and 24 h of infection and calculated as ratio of number of viable cells compared to the total cell numbers and normalised on the viability of uninfected control group. Values are the mean ± SD of three individual experiments. **c** Fold changes of IL-6 and IL-8 mRNA expression were calculated relatively to non-infected controls and normalized to housekeeper. Experiments were performed in triplicate and the mean ± SD is presented. Only altered mRNA expression level above or below the dotted lines are defined as regulated. Asterisks indicate statistically significant differences between tested strains and negative controls at each time point. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$, unpaired *t*-test

Cytokine induction by *C. jejuni* mutants in HT-29/B6 cells

To assess the effects of mutations on the induction of pro-inflammatory cytokines, the mRNA expression of IL-8 and IL-6 was evaluated by calculating the relative fold difference to non-infected cells (controls). The results (Fig. 3c) indicated that all strains uniformly induce IL-8 mRNA expression of host cells at 1 h p.i. followed by

a decrease until 24 h p.i. in a similar manner. Although individual mRNA patterns of IL-6 have been observed during the infection of all strains, no significant differences were observed.

Discussions

Growing evidence has shown that adhesion and invasion is mediated by multiple factors that are critical for pathogenesis of *Campylobacter* [1, 4, 5, 11]. However, the molecular mechanisms of pathogenicity are not fully understood. To continue identifying unknown virulence genes, a transposon mutant library of *C. jejuni* NCTC 11168 was constructed and the ability of individual mutants to adhere to and invade human intestinal epithelial cells was evaluated compared to the wild type. We identified two mutants of *C. jejuni* possessing altered phenotypes with transposon insertions in the genes Cj1492c and Cj1507c.

Gene Cj1492c, annotated as a two-component sensor belongs to the class of Per-Arnt-Sim motif (PAS) domain-containing sensor histidine kinases, for which binding of a chemically diverse range of small-molecule metabolites is typical [31, 32]. It has been reported that mutagenesis of gene Cjj1484 (the orthologue of Cj1492c in *C. jejuni* 81–176) was accompanied with the repression of several genes involved in metabolism, iron/heme acquisition and respiration during cultivation in broth, while no influence on colonisation of chickens was determined [33]. Others have reported that the expression of Cj1492c was up-regulated after treatment with 0.1% sodium deoxycholate and down-regulated after treatment with epinephrine and norepinephrine [34, 35]. However, neither the specific signalling molecule nor the function have yet been clarified.

The gene Cj1507c is annotated as a regulatory protein belonging to the family of LysR transcriptional regulators [22]. But little is known about the functions of LysR family regulators in *Campylobacter*. Another LysR regulator (Cj1000) is reported to facilitate adaption of *C. jejuni* to host colonisation and respiration [36]. Taveirne et al. [22] described that Cj1507c is involved in the repression of the molybdenum transport system *modABC* as well as the tungsten transport system *tunABC* and therefore termed as *modE* [22]. In contrast to other ModE proteins, the *C. jejuni* ModE does not contain a metal binding domain but instead a protein–protein binding site. This indicates that ModE could be a DNA-binding subunit of a multiprotein complex involved in the repression of both transport system operons [22]. Additionally, it has been reported that molybdenum plays an important role during intestinal colonisation of mice by *C. jejuni* 81-176 [37].

Previous studies determined that *C. jejuni* motility is necessary for colonising the mucous layer of the gastrointestinal tract and therefore is important for *C. jejuni* invasion of epithelial cells [1, 4, 5, 8, 18, 38]. The roles of motility, adhesion, invasion or their mutual interplay during pathogenesis have been partially clarified. It is worth noting that both mutants ::Cj1492c and ::Cj1507c retain slightly reduced motility but behave in a reverse manner in terms of adhesion and invasion. While mutagenesis of Cj1507c caused a severe reduction in adhesion and invasion, the disruption of Cj1492c contributed to a significantly enhanced adhesion and invasion. Therefore, it seems unlikely that the slightly reduced motility is the reason for the divergent effects observed for both mutants.

Furthermore, genes involved in intracellular survival have been reported to be also involved in invasion [18, 39]. In order to detect whether mutations of genes Cj1492c and Cj1507c caused altered intracellular survival, we determined the CFU of intracellular *C. jejuni* after 5 and 24 h p.i.. In our experiments, the amount of culturable intracellular bacteria was severely decreased after 24 h for both mutants and wild type. An accentuated difference (one-tenth) in the survival rate between ::Cj1507c and the wild type was found and ::Cj1492c showed intermediate survival (one-half) compared to the wild type. Survival mechanisms of *C. jejuni* in eukaryotic cells are still not fully understood. It has been reported that *C. jejuni* undergoes intracellular metabolic reprogramming upon internalisation contributing to subsequently impaired intracellular persistence [40, 41]. Hence, we assume that both genes Cj1492c and Cj1507c are also involved in the physiological adaptation of *C. jejuni* to the hostile intracellular environment.

Numerous cytotoxins produced by *C. jejuni* might be engaged in infection of host cells. Therefore, we addressed potential correlations between adhesion and invasion along with the impact of tested strains on host cell survival in HT-29/B6 cells after infection. In fact, we observed that there was a small but non-significant decline in the number of viable host cells between infected and non-infected samples. However, no difference was found among wild type and two mutants neither at 5 nor at 24 h p.i.. As the wild type did not impair host cell viability in the time course of our experiments, which might be due to the short period of infection, we cannot exclude potential effects of mutations on host cell cytotoxicity upon infection at later time points.

Similarly, to what had been previously published, we observed significantly enhanced levels of IL-8 mRNA in all infected samples compared to the non-infected controls [42–44]. After an early peak, there was a decrease in expression of IL-8, which agrees with previous observations regarding a down-regulation of pro-inflammatory cytokines after early peak to protect epithelial cells from

chronic inflammation and epithelial destruction [11]. We found that IL-8 expression in response to infection was similar among tested strains throughout the infection course. These data suggest that genes Cj1492c and Cj1507c are not responsible for the induction of IL-8 expression in host cells. Furthermore, the IL-8 mRNA induction in our experiments appears to be independent from capabilities to adhere and to invade host cells or the intracellular persistence. No significant regulation of IL-6 mRNA expression was induced by both mutants and wild type along the entire infection course. According to a previous study, IL-8 is important for extracellular response, whereas IL-6 is considered as an important factor for integrity of epithelial cells and plays a vital role on the intracellular response [45].

The strategies of *C. jejuni* to invade epithelial cells are known as paracellular- and transcellular-pathways [7, 8]. In our immunofluorescent detection, several *C. jejuni* seemed to be located in the intercellular spaces. This might indicate that *C. jejuni* paracellularly transmigrate via cell junctions and then invade the epithelial cells at the basolateral membrane, since the CadF and FlpA binding-protein fibronectin is predominantly located on the basal site of enterocytes [9]. However, by the method applied in our study, we could not exactly distinguish whether the transmigration of tested strains and the infection of HT-29/B6 cells occurred via the trans- or paracellular pathway.

The observed phenotypical differences of mutants might also result from polar effects of the mutation. For the downstream located cognate response regulator of the two-component sensor Cj1492c, it has been reported that it influences expression of some specific genes independently of the histidine kinase Cjj1484 [33]. The gene upstream of Cj1492c encodes an integral membrane protein, containing a conserved protein domain of the possible sulfite exporter TauE/SafE family, which has not been well characterised in *C. jejuni* thus far. The downstream gene of Cj1507c encodes the chemoreceptor Tlp1, responsible for sensing aspartate [46], and mutation of *tlp1* resulted in enhanced adhesion and invasion of CaCo2 cells [47]. The gene *fdhD*, located upstream of Cj1507c, encodes an accessory protein of the formate dehydrogenase, which also has not been well investigated in *C. jejuni*. However, it has been demonstrated that mutation of *fdhD* resulted in similar colonisation abilities but reduced immunopathological responses in mice [48]. Nevertheless, it still remains unclear whether potential polar effects generated by mutagenesis of Cj1492c and Cj1507c play a role or not.

Conclusion

We conclude that genes regulated by Cj1507c have an impact on efficient adhesion, invasion and intracellular survival of *C. jejuni* in HT-29/B6 cells. Furthermore, signal sensing by Cj1492c seems to lead to limiting attachment and hence internalisation of *C. jejuni*. However, as the intracellular survival capacities are reduced, we suggest that signal sensing by Cj1492c may impact several processes related to pathogenicity of *C. jejuni* in HT-29/B6 cells. A more complete understanding of the function of Cj1492c and Cj1507c as well as the regulatory networks is subject of our future investigations.

Methods

Bacterial strains and culture conditions

All bacterial strains used in this study (*C. jejuni* NCTC 11168 and mutants ::Cj1492c, ::Cj1507c and ::*flaA*) were routinely grown on Mueller–Hinton agar (Oxoid, Munich, Germany) supplemented with 5% defibrinated sheep blood (MHA) or in Brucella broth (BB; BD, Heidelberg, Germany) at 37 ℃ under microaerobic conditions (10% CO_2, 6% O_2 and 85% N_2) generated by an Anoxomat (Omni Life Science, Bremen, Germany) as described previously [49]. For invasion, adhesion and infection assays, *C. jejuni* strains were grown to mid-exponential phase (approx. 20 h) in BB at 37 °C under microaerobic conditions and harvested by centrifugation (14,000*g*, 5 min). Cell pellets were resuspended in appropriate buffers or media for further experiments. All in vitro infections with bacteria were performed at a multiplicity of infection (MOI) of 500.

Random transposon mutagenesis

Campylobacter jejuni NCTC 11168 (wild type) was incubated for 24 h in BB at 37 ℃ under microaerobic conditions. DNA was extracted from this culture with Easy-DNA-Extraction kit (Invitrogen, Thermo Fisher Scientific, Waltham, US) according to manufacturer's instructions. The extracted DNA was used for further insertion of HyperMu <KAN1> transposon (Epicentre, Madison, US). Briefly, a total volume of 40 µl containing 5 µg DNA, 1× reaction buffer, 50 ng Transposon and 2 U Transposase were incubated for 2 h at 37 ℃. Reaction was stopped by adding the stop solution and incubation at 70 ℃ for 10 min and was stored at −20 ℃ for further use. Wild type was mutated by natural transformation. In brief, *C. jejuni* NCTC 11168 was incubated in BB for 24 h at 37 ℃ under microaerobic conditions and diluted to $OD_{600} = 0.1$. MHA in glass tubes was overlaid with 0.5 ml of this culture and incubated for 3 h. After addition of 5 µg genomic DNA with integrated transposons, the culture was further incubated for 20 h at 37 ℃ under microaerobic conditions. This culture was streaked

on MHA plates containing 20 µg/ml Kanamycin (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 72 h to select transformants. Each grown colony was enriched by 48 h incubation on MHA containing 50 µg/ml Kanamycin. Insertion site was determined by sequencing with the primers included in the HyperMu <KAN> Transposon Kit and further verified by PCR amplification with gene specific primers. All primers used in this study are listed in Table 1.

Primers used for partial amplification were designed with Primer3 version 0.4.0 [50] based on whole-genome sequence of *C. jejuni* NCTC 11168 [32]. To verify the mutated genes 0.4 µM of each primer, 1× PCR buffer (Qiagen, Hilden, Germany), 1.5 mM $MgCl_2$ (Qiagen), 0.4 mM of each dNTPs (Fermentas, St. Leon-Rot, Germany), 0.5 U Taq-Polymerase (Qiagen) and 2 µl extracted genomic DNA of each mutant were used in PCR. The following amplification protocol was used: initial denaturation for 5 min at 95 ℃ followed by 35 cycles with 30 s at 94 ℃, 60 s at 59 ℃ and 60 s at 72 ℃. Afterwards, a final elongation step was performed for 7 min at 72 ℃. PCR amplification products were separated by 1.5% agarose gel-electrophoresis and fragment size was determined according to the Hyperladder 1 kb Plus (Bioline, Luckenwalde, Germany) standards.

Growth curve

Growth curves of wild type, ::Cj1492c and ::Cj1507c were generated as previously described with few modifications [49]. Briefly, 5 ml BB was inoculated with freshly grown bacteria of each strain and incubated overnight (approximately 18 h) at 37 ℃ under microaerobic conditions. These pre-cultures were diluted in BB to reach a starting culture with approximately 6×10^3 CFU/ml and incubated at 37 ℃ for 48 h under microaerobic conditions. Viable bacterial numbers were determined at indicated time points by plating serial dilutions (10^{-1}) on MHA plates. Before determining the CFU, plates were incubated at 37 ℃ under microaerobic conditions for 48 h. Experiments were repeated independently at least three times and CFU/ml was determined in duplicate.

Swarming assay

Swarming assays were performed according to previous studies [6, 49] with some modifications. The wild type and the mutant strains (::*flaA*, ::Cj1492c, ::Cj1507c) were cultured in BB for 20 h at 37 °C under microaerobic conditions and adjusted to 1×10^8 CFU/ml. 1 µl of each overnight culture was stabbed into Mueller–Hinton plates containing 0.4% agar (MH) and incubated microaerobically at 37 ℃ for 24 h. To minimise plate-to-plate variations, all tested mutant strains and the wild type were included on the same agar plate. The diameter of formed halo of the mutants was measured and normalised to the wild type from the same plate. Each strain was analysed performing at least five independent assays.

Cultivation of human epithelial cells

The sub clone HT-29/B6 [19] of the human colorectal adenocarcinoma cell line HT-29 (DSMZ_ACC 299) was routinely cultured as described previously [51]. Briefly, HT-29/B6 cells were cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v)

Table 1 Primers used in this study

Gene	Primer	Sequence
Cj1492c	Cj1492c_fw	TTT GAT TGT GAG TAA ATT TCA GCA A
	Cj1492c_rev	ATG CAA ATT GCC TTG GAA AA
Cj1507c	Cj1507c_fw	TCG GCT AAT TGT CCG ATT TT
	Cj1507c_rev	CGC TTT GTG GTT TTG CTA GA
flaA	flaA_fw	ACG ATA TAG CAT TTA ACA AG
	flaA_rev	AAC AAC TGA ATT TGC ATG TGC
Transposon	MUKAN-1 FP-1 forward primer	CTG GTC CAC CTA CAA CAA AGG
	MUKAN-1 RP-1 reverse primer	AGA GAT TTT GAG ACA GGA TCC G
IL-6	hsa_IL6_fw	GAA AGC AGC AAA GAG GCA CT
	hsa_IL6_rev	TTT TCA CCA GGC AAG TCT CC
IL-8	hsa_IL8_fw	GTG CAG TTT TGC CAA GGA GT
	hsa_IL8_rev	CTC TGC ACC CAG TTT TCC TT
ACTB	hsa_ACTB_fw	GGA CTT CGA GCA AGA GAT GG
	hsa_ACTB_rev	AGC ACT GTG TTG GCG TAC AG
B2M	hsa_B2M_fw	GTG CTC GCG CTA CTC TCT CT
	hsa_B2M_rev	GGA TGG ATG AAA CCC AGA CA

FCS superior (Biochrom, Cambridge, United Kingdom) in 75 cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany) at 37 ℃ and 5% CO_2 under a humidified atmosphere until a confluence of approx. 80–90% was reached. For adhesion and invasion assays, 1×10^5 HT-29/B6 cells were seeded into each well of a 24 well plate (Sarstedt) and incubated for 7 days changing the media every 3 days. For examining cytotoxicity, each well of a 96 well plate (Sarstedt) was seeded with 1.8×10^4 HT-29/B6 cells and incubated as described above. For gene expression analysis, infection assays were performed in 6 well plates (Sarstedt) with 5×10^5 HT-29/B6 cells seeded per well and following incubation at 37 ℃ and 5% CO_2 routinely changing the medium for 7 days.

In vitro adhesion and invasion assay

Adhesion and invasion assays with the cell line HT-29/B6 were performed with wild type, ::Cj1492c and ::Cj1507c as previously described with slight modification [51, 52]. Briefly, approximately 1×10^5 HT-29/B6 cells were seeded into each well of a 24 well plate. After 7 days of differentiation, cells with ~ 80–90% confluence were infected with a suspension of approximately 1×10^9 CFU of bacteria (MOI 500) for both adhesion and invasion assays. To determine the adhesion of each strain, monolayers were infected for 1 h and incubated at 37 ℃ with 5% CO_2. Afterwards, infected cells were rinsed with phosphate buffered saline (PBS, Sigma-Aldrich) to wash off loosely attached *C. jejuni* and then lysed with 1% Triton-X-100 (Carl Roth, Karlsruhe, Germany) for 10 min at room temperature. For invasion assays, infected monolayers were incubated for 3 h and subsequently treated with 300 ng/ml gentamicin for 2 h (Biochrom) to kill extracellular bacteria. Following additional washes with PBS, cells were lysed as described above. To evaluate numbers of adherent or invasive bacteria, *C. jejuni* from respective lysates were serially diluted and CFU counted on MHA after 48 h incubation at 37 ℃ under microaerobic conditions. Adhesion and invasion index were calculated as percentage of the inoculum, respectively. Each experiment was performed using at least three biological replicates considering three technical replicates for each.

Immunofluorescence detection and microscopy

HT-29/B6 cells were grown on 8-well chamber slides (Sarstedt) to ~ 80–90% confluence and then infected with ::Cj1492c, ::Cj1507c and the wild type (MOI 500) for 3 h and subsequently treated with gentamicin as described above. After incubation, the slides were washed three times with PBS and fixed in 3.7% formaldehyde at room temperature for 15 min. Fixed cells were washed with PBS three times and permeabilised with 0.25% Triton X-100 for 15 min at room temperature followed by three washing steps with PBS. Non-specific binding was blocked with Endogenous Avidin/Biotin blocking kit (Abcam, Cambridge, United Kingdom) according to the manufacturer's protocol followed by incubation in 1% bovine serum albumin (Sigma-Aldrich) in PBST (PBS + 1% Tween 20) for 1 h at room temperature. Cells were subsequently incubated with 1:1000 diluted primary antibody for *C. jejuni* (Biotin-rabbit polyclonal, Abcam) in PBST supplemented with 1% BSA in a humidified chamber overnight at 4 ℃. After three washes with PBS for 5 min, primary antibody was visualised using Alexa Fluor 488 conjugated streptavidin (1:400 diluted) (Thermo Fisher Scientific) following the method previously described with slight modifications [21]. HT-29/B6 cell membranes were subsequently stained with wheat germ agglutinin conjugate WGA-CF®594 (Biotium, Fremont, US) at a concentration of 5 µg/ml in Hank's Balanced Salt Solution (HBSS; Lonza) for 10 min at room temperature, followed by washing twice in PBS. Nuclei were stained with DAPI (200 ng/ml) (Sigma) by 15 min incubation at room temperature. After two washes with PBS, the slides were mounted with 50% glycerol in PBS before fluorescence microscopy using a Leica DMI6000 (Leica, Wetzlar, Germany). The internalisation level of tested strains was calculated as a ratio of invaded *C. jejuni* and infected host cells, which were automatically counted by the ImageJ software as previously described [27]. Briefly, we used ImageJ to outline interested signals by thresholding and then identified particles with a specific size range. Afterwards, the particle counting was automatically measured by the Region of Interest (RoI) manager.

Cell viability determination

To study effects of *C. jejuni* on HT-29/B6 viability, Calcein-acetoxymethyl/Hoechst staining was performed. Calcein-acetoxymethyl (Calcein-AM; Biotium) has already been described as a green fluorescent indicator of viable cells in cytotoxicity assays [29, 30]. Hoechst 33342 (Thermo Fisher Scientific) (hereinafter referred to as Hoechst) is used as a blue fluorescent marker of the nuclei of all cells [53]. Viability of HT-29/B6 cells was investigated in 96 well plates (Sarstedt). After 7 days differentiation, HT-29/B6 with ~ 80–90% confluence were infected with wild type, ::Cj1492c and ::Cj1507c for 5 h and 24 h as described before. Infected monolayers were washed 3 times with PBS and incubated with 0.4 µM Calcein-AM at 37 ℃ with 5% CO_2 for 30 min. 5 µg/ml Hoechst was added to each well and incubated 15 min at room temperature followed by two washes with PBS. Fluorescence micrographs were subsequently captured with a Leica DMI6000 (Leica). The relative viability of

host cells was calculated as percentage of the number of viable cells compared to the total cell numbers automatically determined using the ImageJ software as described above [27]. Presented results are calculated from three individual assays.

RNA isolation and RT-qPCR

For mRNA expression analysis, in vitro infection was performed as described above and HT-29/B6 samples were taken at 1, 5 and 24 h p.i. considering respective negative controls (non-infected cells). For this, cell monolayers were washed three times with PBS and lysed directly in each well of 8-well plates by addition of cell lysis buffer (Roboklon, Berlin, Germany). Total RNA was isolated with Universal RNA/miRNA Purification Kit (Roboklon) according to the manufacturer's protocol. The RNA quality and quantity was controlled using the Agilent 2100 Bioanalyzer with the RNA Nano Chips (Agilent, Waldbronn, Germany) and the DS-11 Spectrophotometer/Fluorometer (DeNovix Inc., Wilmington, USA), respectively. Relative expression of mRNA coding for IL-6 and IL-8 was quantified by RT-qPCR as described earlier [54]. For normalisation of gene expression, ACTB and B2M transcripts were used as reference genes and relative gene expression was calculated as described earlier [54] by the ΔΔCt method [55]. Experiments were performed in triplicate. Primers used in this study are listed in Table 1.

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Authors' contributions
DX Performed experiments, analysed data, wrote paper. TA Provided advice in study design, critically discussed results, co-edited paper. RE Provided advice in study design, co-edited paper. SS and GG Designed study, performed experiments, analysed data, co-wrote paper. All authors read and approved the final manuscript.

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References

1. Bolton DJ. *Campylobacter* virulence and survival factors. Food Microbiol. 2015;48:99–108.
2. Freitag CM, Strijbis K, van Putten JPM. Host cell binding of the flagellar tip protein of *Campylobacter jejuni*. Cell Microbiol. 2017;19(6):e12714.
3. Bouwman LI, de Zoete MR, Bleumink-Pluym NM, Flavell RA, van Putten JP. Inflammasome activation by *Campylobacter jejuni*. J Immunol. 2014;193(9):4548–57.
4. Dasti JI, Tareen AM, Lugert R, Zautner AE, Gross U. *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. Int J Med Microbiol. 2010;300(4):205–11.
5. van Vliet AH, Ketley JM. Pathogenesis of enteric Campylobacter infection. Symp Ser. 2001;30:45S–56S.
6. Fearnley C, Manning G, Bagnall M, Javed MA, Wassenaar TM, Newell DG. Identification of hyperinvasive *Campylobacter jejuni* strains isolated from poultry and human clinical sources. J Med Microbiol. 2008;57(Pt 5):570–80.
7. Backert S, Boehm M, Wessler S, Tegtmeyer N. Transmigration route of *Campylobacter jejuni* across polarized intestinal epithelial cells: paracellular, transcellular or both? Cell Commun Signal. 2013;11:72.
8. O Cróinín T, Backert S. Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism? Front Cell Infect Microbiol. 2012;2:25.
9. Harrer A, Bucker R, Boehm M, Zarzecka U, Tegtmeyer N, Sticht H, Schulzke JD, Backert S. *Campylobacter jejuni* enters gut epithelial cells and impairs intestinal barrier function through cleavage of occludin by serine protease HtrA. Gut Pathog. 2019;11:4.
10. Javed MA, Grant AJ, Bagnall MC, Maskell DJ, Newell DG, Manning G. Transposon mutagenesis in a hyper-invasive clinical isolate of *Campylobacter jejuni* reveals a number of genes with potential roles in invasion. Microbiology. 2010;156(Pt 4):1134–43.
11. Aguilar C, Jimenez-Marin A, Martins RP, Garrido JJ. Interaction between Campylobacter and intestinal epithelial cells leads to a different proinflammatory response in human and porcine host. Vet Immunol Immunopathol. 2014;162(1–2):14–23.
12. Rubinchik S, Seddon A, Karlyshev AV. Molecular mechanisms and biological role of *Campylobacter jejuni* attachment to host cells. Eur J Microbiol Immunol (Bp). 2012;2(1):32–40.
13. Hickey TE, McVeigh AL, Scott DA, Michielutti RE, Bixby A, Carroll SA, Bourgeois AL, Guerry P. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. Infect Immun. 2000;68(12):6535–41.
14. Rodrigues RC, Pocheron AL, Cappelier JM, Tresse O, Haddad N. An adapted in vitro assay to assess *Campylobacter jejuni* interaction with intestinal epithelial cells: taking into stimulation with TNFalpha. J Microbiol Methods. 2018;149:67–72.
15. Bahrami B, Macfarlane S, Macfarlane GT. Induction of cytokine formation by human intestinal bacteria in gut epithelial cell lines. J Appl Microbiol. 2011;110(1):353–63.
16. Hickey TE, Baqar S, Bourgeois AL, Ewing CP, Guerry P. *Campylobacter jejuni*-stimulated secretion of interleukin-8 by INT407 cells. Infect Immun. 1999;67(1):88–93.
17. Mellits KH, Mullen J, Wand M, Armbruster G, Patel A, Connerton PL, Skelly M, Connerton IF. Activation of the transcription factor NF-kappaB by *Campylobacter jejuni*. Microbiology. 2002;148(Pt 9):2753–63.
18. Backert S, Hofreuter D. Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen *Campylobacter jejuni*. J Microbiol Methods. 2013;95(1):8–23.
19. Kreusel KM, Fromm M, Schulzke JD, Hegel U. Cl-secretion in epithelial monolayers of mucus-forming human colon cells (HT-29/B6). Am J Physiol. 1991;261(4 Pt 1):C574–82.
20. Wilson DL, Bell JA, Young VB, Wilder SR, Mansfield LS, Linz JE. Variation of the natural transformation frequency of *Campylobacter jejuni* in liquid shake culture. Microbiology. 2003;149(Pt 12):3603–15.

21. Stahl M, Ries J, Vermeulen J, Yang H, Sham HP, Crowley SM, Badayeva Y, Turvey SE, Gaynor EC, Li X, et al. A novel mouse model of *Campylobacter jejuni* gastroenteritis reveals key pro-inflammatory and tissue protective roles for toll-like receptor signaling during infection. PLoS Pathog. 2014;10(7):e1004264.
22. Taveirne ME, Sikes ML, Olson JW. Molybdenum and tungsten in *Campylobacter jejuni*: their physiological role and identification of separate transporters regulated by a single ModE-like protein. Mol Microbiol. 2009;74(3):758–71.
23. Konkel ME, Klena JD, Rivera-Amill V, Monteville MR, Biswas D, Raphael B, Mickelson J. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. J Bacteriol. 2004;186(11):3296–303.
24. Novik V, Hofreuter D, Galán JE. Characterization of a *Campylobacter jejuni* VirK protein homolog as a novel virulence determinant. Infect Immun. 2009;77(12):5428–36.
25. Lertsethtakarn P, Ottemann KM, Hendrixson DR. Motility and chemotaxis in Campylobacter and Helicobacter. Annu Rev Microbiol. 2011;65(1):389–410.
26. Goldstein IJ, Winter HC, Poretz RD. Chapter 12—plant lectins: tools for the study of complex carbohydrates. In: Montreuil J, Vliegenthart JFG, Schachter H, editors. New comprehensive biochemistry, vol. 29. New York: Elsevier; 1997. p. 403–74.
27. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671–5.
28. Pawar K, Sharbati J, Einspanier R, Sharbati S. Mycobacterium bovis BCG interferes with miR-3619-5p control of cathepsin S in the process of autophagy. Front Cell Infect Microbiol. 2016;6:27.
29. Papadopoulos NG, Dedoussis GVZ, Spanakos G, Gritzapis AD, Baxevanis CN, Papamichail M. An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. J Immunol Methods. 1994;177(1):101–11.
30. Roden MM, Lee K-H, Panelli MC, Marincola FM. A novel cytolysis assay using fluorescent labeling and quantitative fluorescent scanning technology. J Immunol Methods. 1999;226(1):29–41.
31. Henry JT, Crosson S. Ligand-binding PAS domains in a genomic, cellular, and structural context. Annu Rev Microbiol. 2011;65:261–86.
32. Parkhill J, Wren BW, Mungall K, Ketley JM, Churcher C, Basham D, Chillingworth T, Davies RM, Feltwell T, Holroyd S, et al. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. Nature. 2000;403(6770):665–8.
33. Luethy PM, Huynh S, Parker CT, Hendrixson DR. Analysis of the activity and regulon of the two-component regulatory system composed by Cjj81176_1484 and Cjj81176_1483 of *Campylobacter jejuni*. J Bacteriol. 2015;197(9):1592–605.
34. Malik-Kale P, Parker CT, Konkel ME. Culture of *Campylobacter jejuni* with sodium deoxycholate induces virulence gene expression. J Bacteriol. 2008;190(7):2286–97.
35. Xu F, Wu C, Guo F, Cui G, Zeng X, Yang B, Lin J. Transcriptomic analysis of *Campylobacter jejuni* NCTC 11168 in response to epinephrine and norepinephrine. Front Microbiol. 2015;6:452.
36. Dufour V, Li J, Flint A, Rosenfeld E, Rivoal K, Georgeault S, Alazzam B, Ermel G, Stintzi A, Bonnaure-Mallet M, et al. Inactivation of the LysR regulator Cj1000 of *Campylobacter jejuni* affects host colonization and respiration. Microbiology. 2013;159(Pt 6):1165–78.
37. Gao B, Vorwerk H, Huber C, Lara-Tejero M, Mohr J, Goodman AL, Eisenreich W, Galan JE, Hofreuter D. Metabolic and fitness determinants for in vitro growth and intestinal colonization of the bacterial pathogen *Campylobacter jejuni*. PLoS Biol. 2017;15(5):e2001390.
38. Young KT, Davis LM, Dirita VJ. *Campylobacter jejuni*: molecular biology and pathogenesis. Nat Rev Microbiol. 2007;5(9):665–79.
39. Novik V, Hofreuter D, Galán JE. Identification of *Campylobacter jejuni* genes involved in its interaction with epithelial cells. Infect Immun. 2010;78(8):3540–53.
40. Pryjma M, Apel D, Huynh S, Parker CT, Gaynor EC. FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of *Campylobacter jejuni* following host cell infection. J Bacteriol. 2012;194(15):3803–13.
41. Watson RO, Galan JE. *Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes. PLoS Pathog. 2008;4(1):e14.
42. Lai CK, Chen YA, Lin CJ, Lin HJ, Kao MC, Huang MZ, Lin YH, Chiang-Ni C, Chen CJ, Lo UG, et al. Molecular mechanisms and potential clinical applications of *Campylobacter jejuni* cytolethal distending toxin. Front Cell Infect Microbiol. 2016;6:9.
43. MacCallum AJ, Harris D, Haddock G, Everest PH. *Campylobacter jejuni*-infected human epithelial cell lines vary in their ability to secrete interleukin-8 compared to in vitro-infected primary human intestinal tissue. Microbiology. 2006;152(Pt 12):3661–5.
44. Chen ML, Ge Z, Fox JG, Schauer DB. Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*. Infect Immun. 2006;74(12):6581–9.
45. Gundogdu O, Mills DC, Elmi A, Martin MJ, Wren BW, Dorrell N. The *Campylobacter jejuni* transcriptional regulator Cj1556 plays a role in the oxidative and aerobic stress response and is important for bacterial survival in vivo. J Bacteriol. 2011;193(16):4238–49.
46. Korolik V. Aspartate chemosensory receptor signalling in *Campylobacter jejuni*. Virulence. 2010;1(5):414–7.
47. Hartley-Tassell LE, Shewell LK, Day CJ, Wilson JC, Sandhu R, Ketley JM, Korolik V. Identification and characterization of the aspartate chemosensory receptor of *Campylobacter jejuni*. Mol Microbiol. 2010;75(3):710–30.
48. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kuhl AA, Dasti JI, Zautner AE, Munoz M, Loddenkemper C, et al. Novel murine infection models provide deep insights into the "menage a trois" of *Campylobacter jejuni*, microbiota and host innate immunity. PLoS ONE. 2011;6(6):e20953.
49. Adler L, Alter T, Sharbati S, Golz G. Phenotypes of *Campylobacter jejuni* luxS mutants are depending on strain background, kind of mutation and experimental conditions. PLoS ONE. 2014;9(8):e104399.
50. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000;132:365–86.
51. Karadas G, Sharbati S, Hanel I, Messelhausser U, Glocker E, Alter T, Golz G. Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*. J Appl Microbiol. 2013;115(2):583–90.
52. Bruegge J, Hanisch C, Einspanier R, Alter T, Golz G, Sharbati S. *Arcobacter butzleri* induces a pro-inflammatory response in THP-1 derived macrophages and has limited ability for intracellular survival. Int J Med Microbiol. 2014;304(8):1209–17.
53. Gilbert DF, Erdmann G, Zhang X, Fritzsche A, Demir K, Jaedicke A, Muehlenberg K, Wanker EE, Boutros M. A novel multiplex cell viability assay for high-throughput RNAi screening. PLoS ONE. 2011;6(12):e28338.
54. Sharbati J, Lewin A, Kutz-Lohroff B, Kamal E, Einspanier R, Sharbati S. Integrated microRNA–mRNA-analysis of human monocyte derived macrophages upon *Mycobacterium avium* subsp. hominissuis infection. PLoS ONE. 2011;6(5):e20258.
55. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25(4):402–8.

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The glycosyltransferase ST3GAL2 is regulated by miR-615-3p in the intestinal tract of *Campylobacter jejuni* infected mice

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Abstract

Background: *Campylobacter jejuni* (*C. jejuni*) infections are of increasing importance worldwide. As a typical mucosal pathogen, the interaction of *C. jejuni* with mucins is a prominent step in the colonisation of mucosal surfaces. Despite recent advances in understanding the interaction between bacterial pathogens and host mucins, the mechanisms of mucin glycosylation during intestinal *C. jejuni* infection remain largely unclear. This prompted us to identify relevant regulatory networks that are concerted by miRNAs and could play a role in the mucin modification and interaction.

Results: We firstly used a human intestinal in vitro model, in which we observed altered transcription of MUC2 and TFF3 upon *C. jejuni* NCTC 11168 infection. Using a combined approach consisting of in silico analysis together with in vitro expression analysis, we identified the conserved miRNAs miR-125a-5p and miR-615-3p associated with MUC2 and TFF3. Further pathway analyses showed that both miRNAs appear to regulate glycosyltransferases, which are related to the KEGG pathway 'Mucin type *O*-glycan biosynthesis'. To validate the proposed interactions, we applied an in vivo approach utilising a well-established secondary abiotic IL-10$^{-/-}$ mouse model for infection with *C. jejuni* 81-176. In colonic tissue samples, we confirmed infection-dependent aberrant transcription of MUC2 and TFF3. Moreover, two predicted glycosyltransferases, the sialyltransferases ST3GAL1 and ST3GAL2, exhibited inversely correlated transcriptional levels compared to the expression of the identified miRNAs miR-125a-5p and miR-615-3p, respectively. In this study, we mainly focused on the interaction between miR-615-3p and ST3GAL2 and were able to demonstrate their molecular interaction using luciferase reporter assays and RNAi. Detection of ST3GAL2 in murine colonic tissue by immunofluorescence demonstrated reduced intensity after *C. jejuni* 81-176 infection and was thus consistent with the observations made above.

Conclusions: We report here for the first time the regulation of glycosyltransferases by miRNAs during murine infection with *C. jejuni* 81-176. Our data suggest that mucin type *O*-glycan biosynthesis is concerted by the interplay of miRNAs and glycosyltransferases, which could determine the shape of intestinal glycosylated proteins during infection.

Keywords: *Campylobacter jejuni*, Mucin, miRNA, Glycosyltransferase, Host cell response

Background

The colonic mucosal layer is a dynamic and constantly renewing physical barrier providing protective functions through viscoelastic properties of mucins [1]. MUC2 is characterised by containing repeating domains rich in the amino acids proline, threonine and serine (PTS

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domains), which are densely *O*-glycosylated by the glycosylation machinery of the Golgi apparatus [2]. The PTS domain decorated with abundant *O*-glycans (mucin type *O*-glycans) forms a special "mucin domain" that makes up approximately 80% of the MUC2 mass and gives mucins extended conformation and furthermore, contributes to the structural integrity and complexity of mucins [2]. Assembled MUC2 polymers are stored in a condensed way in granules within goblet cells before being released into intestinal lumen. Once released, these large polymers are expanded in volume to form the mucus gel and provide the structural basis of the mucus layer [2]. Besides the mucins, also mucin-associated proteins such as the trefoil factor family (TFF) peptides are important for the restitution and integrity of the intestinal mucosal layer [3]. TFF3 is co-secreted with MUC2 by colonic goblet cells and usually acts in a cooperative manner with MUC2 ensuring elasticity and viscosity of MUC2 [3, 4].

The mucus layer provides protection from enteropathogens in several ways. This essential physical barrier hinders pathogenic as well as commensal bacteria to access the underlying intestinal epithelium. Previous studies have found that the lack of MUC2 and TFF3 resulted in pronounced defects in mouse intestinal homeostasis and enhanced susceptibility towards colitis, emphasising the importance of the mucus layer [1, 5, 6]. Moreover, the role of glycosylation of MUC2 in protecting the host from intruding bacteria has gained some appreciation in recent years. Application of knockout-based experiments led to reports of several glycosyltransferases involved in the biosynthesis and structural formation of core *O*-glycans of MUC2. For example, mice deficient in *N*-acetylglucosaminyltransferase (GCNT2) displayed an overall altered mucin composition and exhibited a defective mucus barrier function, given that GCNT2 initiates core 2 derived *O*-glycan branching which is the basis of mucin core structure 2 and 4 [7]. This indicates that *O*-glycosylation of MUC2 has important implications in forming the skeleton of the mucus layer and maintaining integrity of mucus barrier. Nevertheless, the mechanisms that regulate post-transcriptional modification of *O*-linked glycans on mucins like sialylation or fucosylation remain still unclear.

O-glycans are oligosaccharide moieties abundant on the cell surface proteins of both host and pathogens forming a critical interface with the biological milieu and profoundly influence the pathogen–host interaction [8, 9]. *O*-glycans on mucin proteins are used as ligands for bacterial adhesins and can prevent bacteria to access epithelial cells by binding and hampering them in mucus [10]. On the other hand, specific binding via "glycan-glycan talk" constitutes an important mechanism for bacteria to mediate adhesion and invasion of host cells [8]. During infection, bacterial pathogens can mediate host cell glycan modification utilising a broad range of glycosyltransferases and glycosidases, which in turn facilitates host adaption and adhesion and promotes access to glycans as carbon sources [8, 11]. *Campylobacter jejuni* (*C. jejuni*) is a major cause of bacterial food-borne disease worldwide and capable to induce gastroenteritis and irritable bowel disease in humans [12]. In the course of colonisation, the binding of *C. jejuni* to the host mucins is mediated by glycan-glycan interaction [8, 11]. Nevertheless, only little is known about the mechanisms of *C. jejuni* interaction with glycans of host mucosal surfaces and corresponding regulatory pathways involving mucin-modifying enzymes such as glycosyltransferases.

Recent efforts in non-coding RNA (ncRNA) research have expanded our understanding of mechanisms that regulate gene expression. MicroRNA (miRNA) belong to a well-studied class of ncRNAs, which can regulate gene expression through binding to complementary target sites of e.g. messenger RNAs (mRNAs), thereby initiating their degradation or suppression of translation [13]. Based on the fact that a single miRNA can target different genes and a single target gene can be coordinately regulated by different miRNAs, complex regulatory networks are formed to concert diverse biological processes including cell differentiation, proliferation, apoptosis or immune response [13, 14]. Our work as well as that of other research groups showed that miRNAs play a significant role in bacterial infections [14–17]. For example, the miR-125 family was shown to play an important role in immune response to bacterial as well as viral infections. Increased expression of miR-125b induced by lipomannan from virulent *Mycobacterium tuberculosis* can destabilise the transcript of tumour necrosis factor and therefore block its biosynthesis [17]. Moreover, work form Zhou et al. indicated that during *Helicobacter pylori* (*H. pylori*) infection, MUC2 expression could be post-transcriptionally affected by the cooperation of the lncRNA AF147447 with miR-34c [18]. Interestingly, Singh et al. found that under the influence of the endogenous microbiota, murine caecal miRNA signatures vary. Thus, intestinal barrier function may be affected by targeting genes that encode junctional proteins or glycosylation enzymes [19]. Furthermore, growing evidence has shown that miRNAs are major regulators of the glycome, controlling the level of glycan biosynthesis enzymes and playing a pivotal role in modulating and controlling glycosylation [20]. Kurcon et al. have utilised the glycogene target network of miR-200-family to identify three glycosylation enzymes (ST3 β-Galactoside α-2,3-Sialyltransferase 5 (ST3GAL5), ST6 *N*-acetyl-galactosaminide α-2,6-Sialyltransferase 5 (ST6GALNAC5) and β1,3-Glucosyltransferase (B3GLCT), controlling

epithelial-to-mesenchymal transition in MDA-MB-231 cells [21]. Nevertheless, the number of validated glycogene-miRNA-interactions is still limited [20].

In this work, we applied in vitro and in vivo approaches to address which intestinal miRNA may be associated with MUC2 modification during *C. jejuni* infection and what potential regulatory networks exist in this context. Firstly, we confirmed that *C. jejuni* NCTC 11168 infection induced an altered transcription of MUC2 and the co-secreted peptide TFF3 in human intestinal epithelial cells in vitro. After in silico prediction of mucin-associated regulatory networks, we secondly examined the expression of two miRNAs and two glycosyltransferase genes in murine colonic tissue and found *C. jejuni* 81-176-mediated dysregulation of their transcription. Thirdly, we focused on the ST3 β-Galactoside α-2,3-Sialyltransferase 2 (ST3GAL2) and miR-615-3p interaction given their distinguished anti-correlated transcription upon *C. jejuni* 81-176 infection in vivo and were able to show that miR-615-3p interacts with ST3GAL2 targeting its cellular concentrations.

Results

C. jejuni infection alters the transcription of MUC2 and TFF3 in human intestinal epithelial cells in vitro

MUC2 and TFF3 expression undergo changes during intestinal diseases caused by pathogenic bacteria, for instance [22]. In order to address this and find associated regulatory networks in response to *C. jejuni* infection, we determined the transcription of MUC2 and TFF3 in the human intestinal epithelial cell line HT-29/B6 following *C. jejuni* NCTC 11168 infection. The relative fold differences of MUC2 and TFF3 mRNA levels were calculated and revealed an approximately 2-fold up-regulation ($P \leq 0.01$) of both factors relatively early (i.e., 1 h) post infection (p.i.) (Fig. 1A and B). Thereafter, a consistent decrease of MUC2 transcription was observed in the course of infection until 24 h p.i., whereas no significant differences could be assessed when comparing the infected and non-infected control groups at 5 and 24 h p.i. (Fig. 1A). Upon infection, the transcript level of TFF3 showed an early peak followed by a 0.61-fold decline at 5 h p.i. and a return to control levels until 24 h p.i. (Fig. 1B). Hence, both MUC2 and TFF3 mRNA expression significantly increased immediately after *C. jejuni* infection and subsequently decreased to levels that were comparable to non-infected controls.

Prediction of MUC2-TFF3-associated miRNAs

Recent data [15, 16] showed that regulatory miRNA networks influence the host response to bacterial infections. Therefore, we speculated that besides the alterations in the transcription of MUC2 and TFF3 also post-translational modification of MUC2 might be changed which might be due to pathogen-mediated manipulation of regulatory networks composed of mRNA and miRNAs. To address this, we used miRmap [23] with a score of above 50—as described previously [24]—to identify miRNAs supposed to target MUC2 and TFF3, respectively. After intersecting both lists of miRNAs, we identified 12 miRNAs (Fig. 1C and Additional file 1) that could mutually target MUC2 and TFF3. From this, we selected eight miRNAs, which were conserved among human, mouse and rat. To assess their involvement in *C. jejuni* infection in vitro, we evaluated the expression of miR-125a-5p, miR-615-3p, miR-671-5p and miR-320 family in *C. jejuni* NCTC 11168 infected HT-29/B6 cells. The RT-qPCR results indicated that only miR-125a-5p and miR-615-3p showed regulated expression within 24 h p.i. Immediately after infection, miR-125a-5p was significantly downregulated (0.72-fold, $P \leq 0.05$), whereas its expression returned to baseline values afterwards (Fig. 1D). MiR-615-3p expression showed a similar regulation with a 0.84-fold decrease ($P \leq 0.05$) and its transcript level was rather consistent until 24 h p.i. (0.83-fold, $P \leq 0.05$) (Fig. 1E). Both miRNAs possessed anti-correlated expression with MUC2 and TFF3 mRNAs. As shown in Fig. 1F, miR-320 expression was barely affected along the entire course of infection. Of note, the miR-671-5p level was below detection limit (data not shown).

Identification of regulatory networks for in vivo analysis

As shown above, an inverse correlation between two predicted miRNAs and MUC2 as well as TFF3 was observed in the early phase of *C. jejuni* NCTC 11168 infection in human cells. This indicated a regulatory importance of miR-125a-5p and miR-615-3p on related pathways upon *C. jejuni* infection. Therefore, we expanded our analysis to determine genes that are relevant to the post-transcriptional modification of MUC2 and might be regulated by the same miRNAs in the context of *C. jejuni* infection in vivo applying secondary abiotic mice [25]. For this purpose, we predicted the murine targets of both miR-125a-5p and miR-615-3p by means of another round of miRmap analysis (Additional file 1). After intersecting both lists of targets, we determined a gene list that was supposed to be regulated by both miRNAs. Using this list, we performed a KEGG pathway enrichment [26] by means of Cytoscape with the app ClueGO [27] (Fig. 2A, Additional file 2). In total, target genes were significantly assigned to seven KEGG terms and we found that four target genes were significantly ($P \leq 0.05$) enriched in the KEGG pathway "mmu00512: namely, Mucin type *O*-glycan biosynthesis", which corresponded to 14.3% of the associated genes in this term. They consisted of ST3 β-Galactoside α-2,3-Sialyltransferase 1 (ST3GAL1), ST3

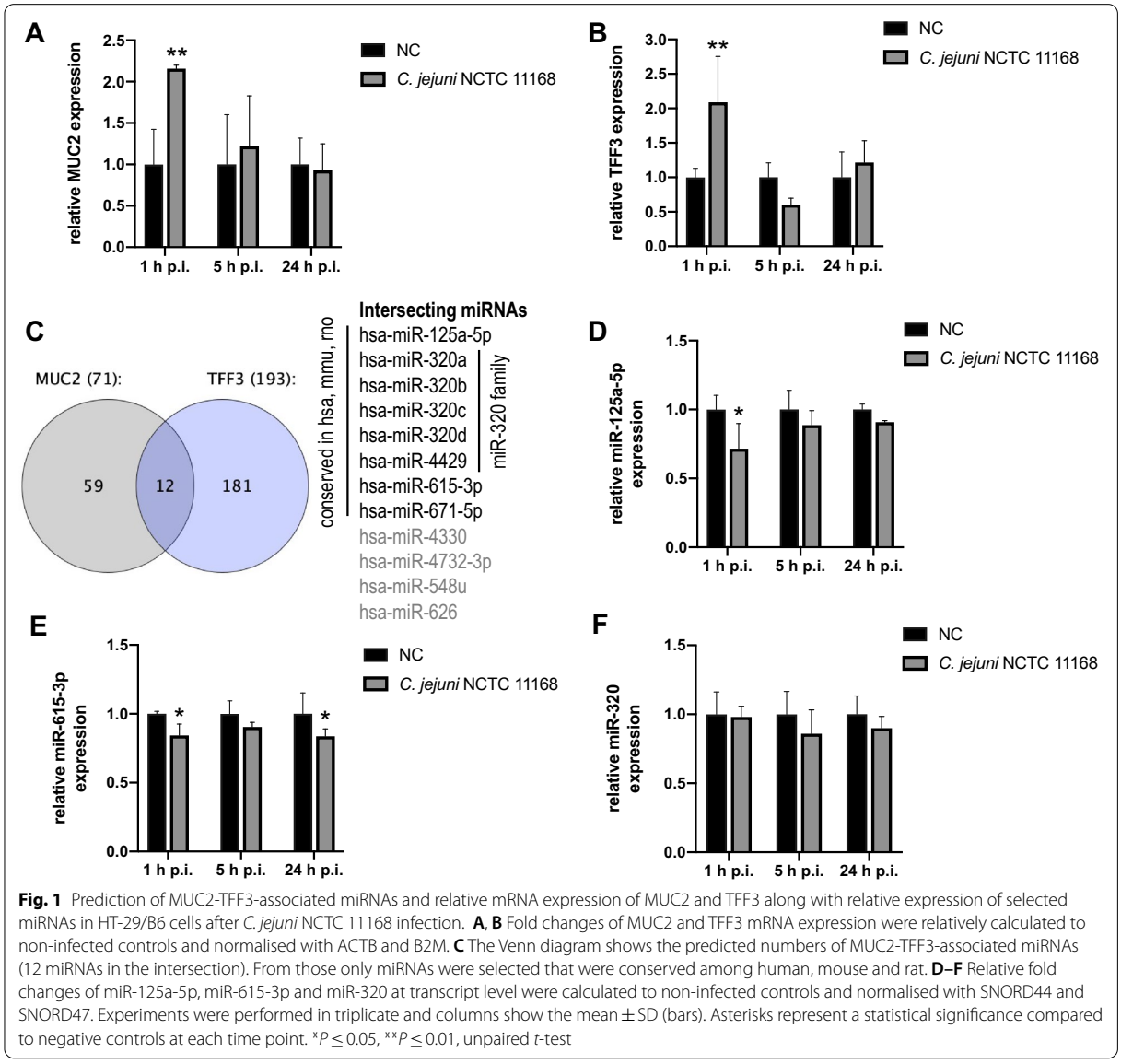

Fig. 1 Prediction of MUC2-TFF3-associated miRNAs and relative mRNA expression of MUC2 and TFF3 along with relative expression of selected miRNAs in HT-29/B6 cells after *C. jejuni* NCTC 11168 infection. **A**, **B** Fold changes of MUC2 and TFF3 mRNA expression were relatively calculated to non-infected controls and normalised with ACTB and B2M. **C** The Venn diagram shows the predicted numbers of MUC2-TFF3-associated miRNAs (12 miRNAs in the intersection). From those only miRNAs were selected that were conserved among human, mouse and rat. **D–F** Relative fold changes of miR-125a-5p, miR-615-3p and miR-320 at transcript level were calculated to non-infected controls and normalised with SNORD44 and SNORD47. Experiments were performed in triplicate and columns show the mean ± SD (bars). Asterisks represent a statistical significance compared to negative controls at each time point. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, unpaired *t*-test

β-Galactoside α-2,3-Sialyltransferase 2 (ST3GAL2), β1,6-*N*-acetylglucosaminyltransferase 1 (GCNT1), and β1,6-*N*-acetylglucosaminyltransferase 4 (GCNT4). These four glycosyltransferases were identified as potentially regulated candidates of both miRNAs upon *C. jejuni* infection in vivo (Fig. 2A).

Expression analysis of interacting miRNAs and mRNAs in the colon of *C. jejuni* infected secondary abiotic IL-10$^{-/-}$ mice

In addition to the early stage of infection with *C. jejuni* NCTC 11168 investigated in human cells, we further surveyed the later stage of infection with *C. jejuni* strain 81-176 in a well-established murine model [25] to assess the biological relevance of the observed regulations and predicted interactions in vivo. Therefore, the relative transcription of MUC2 and TFF3 was measured in colonic tissue samples taken at day 6 following peroral infection of secondary abiotic IL-10$^{-/-}$ mice. As shown in Fig. 2B, *C. jejuni* 81–176 infection resulted in a pronounced decrease of TFF3 transcription when compared to naïve controls (0.38-fold change, $P \leq 0.0001$), whereas the MUC2 transcription was slightly decreased upon infection (0.80-fold

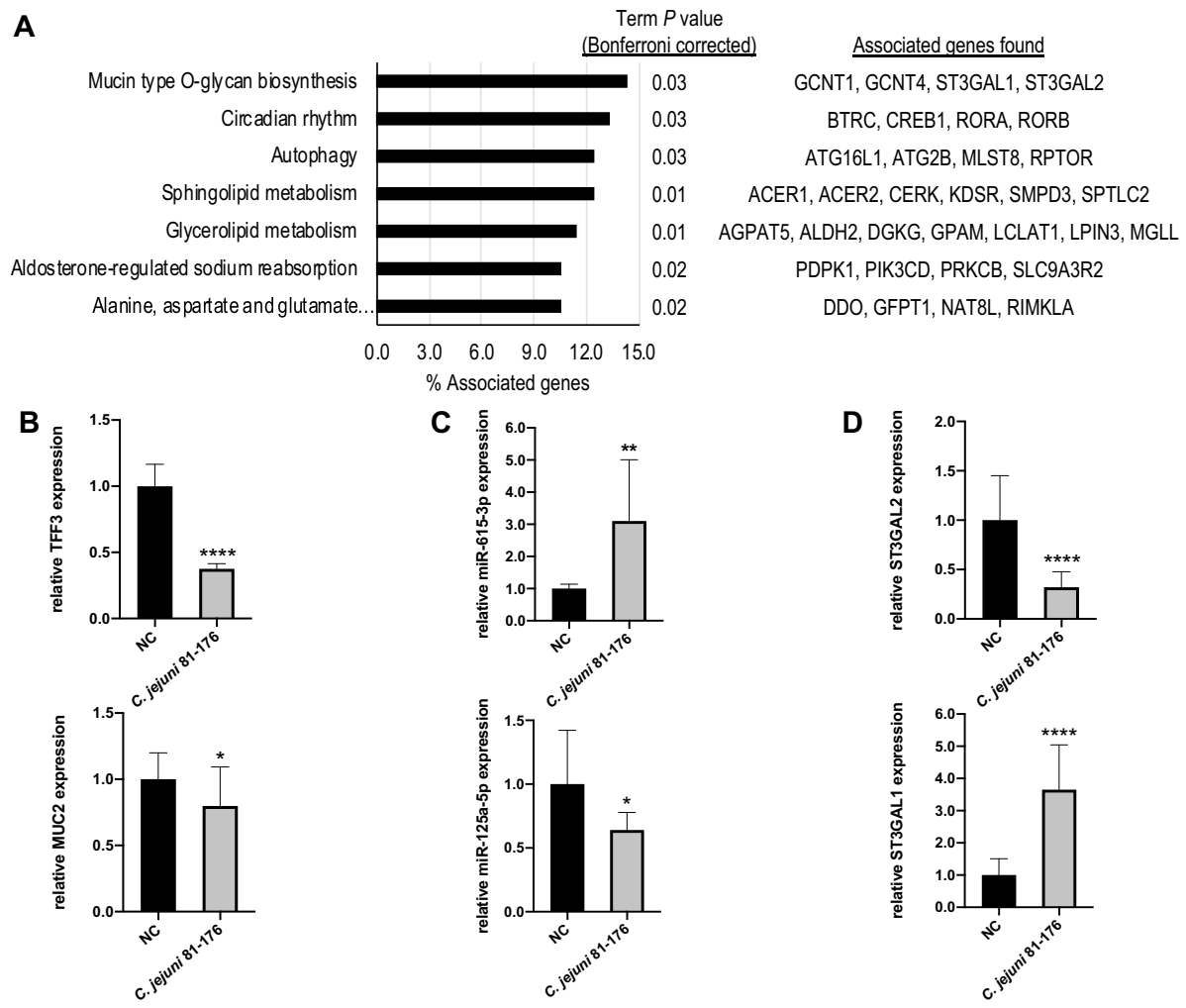

Fig. 2 KEGG pathway enrichment with potential murine targets of miR-125a-5p and miR-615-3p and relative expression of mRNA and miRNA in colonic tissue of secondary abiotic IL-$10^{-/-}$ mice upon *C. jejuni* 81–176 infection. **A** Gene enrichment analysis of mutual target genes of miR-125a-5p and miR-615-3p showing four significantly accumulated target genes (14.3% of associated genes) in the KEGG pathway 'Mucin type *O*-glycan biosynthesis' possessing a term *P* value corrected with Bonferroni step down of 0.03. **B** Fold changes of MUC2 and TFF3 mRNA expression were relatively calculated to naïve controls (NC) and normalised with HPRT and SDHA. **C** Expression levels of miRNA-615-3p and miR-125a-5p were calculated relatively to naïve controls (NC) and normalised with miR-16 and miR-145. **D** The relative transcript levels of ST3GAL1 and ST3GAL2 mRNA were calculated to naïve controls and normalised with HPRT and SDHA. The qPCR data were reported as the average of the relative expression in at least ten biological replicates; columns show the mean ± SD (bars). Asterisks represent a statistical significance compared to negative controls at each time point. *$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq 0.0001$, unpaired *t*-test

change, $P \leq 0.05$). Secondly, we addressed whether the identified miRNAs were also regulated during *C. jejuni* 81-176 infection in mice, especially at the late stage of infection. As demonstrated in Fig. 2C, infected (secondary abiotic IL-$10^{-/-}$) mice exhibited more than triple miR-615-3p transcript levels ($P \leq 0.01$) compared to non-infected control mice. In contrast, the miR-125a-5p transcription decreased at the late stage of *C. jejuni* infection (0.64-fold changes, $P \leq 0.05$; Fig. 2C). As pathway analysis predicted that glycosyltransferases involved in the mucin type *O*-glycan biosynthesis might be regulated by the identified miR-125a-5p and miR-615-3p, we were intrigued to unravel whether predicted targets ST3GAL1 and ST3GAL2 are dysregulated in an anti-correlated manner. Therefore, we examined their transcription in identical samples and

observed a strongly reduced transcription (0.32-fold changes, $P \leq 0.0001$) of ST3GAL2 elicited by *C. jejuni* 81–176 infection (Fig. 2D), which was anti-correlated with the miR-615-3p expression. In contrast, ST3GAL1 transcription was markedly enhanced (3.65-fold, $P \leq 0.0001$) when compared to naïve controls (Fig. 2D) and exhibited inverse correlation with miR-125a-5p.

ST3GAL2 is down-regulated in the murine epithelial cell line CMT 93 after RNAi using miR-615-3p mimic

MiRNAs are known to play a pivotal role in mRNA degradation or transcriptional repression by binding to their targets [13]. The results of the in vivo experiments suggested that the mRNA level of ST3GAL2 might be modulated by miR-615-3p and miR-125a-5p may play a regulatory role on ST3GAL1 transcription in colonic tissue after *C. jejuni* 81–176 infection. In this study, we only focused on the interaction of ST3GAL2 and miR-615-3p. To explore whether miR-615-3p was responsible for the decreased ST3GAL2 mRNA levels, we performed RNAi experiments using miR-615-3p mimics in the mouse rectum cell line CMT 93 considering nonsense transfected cells as controls. After RNAi, expression analysis of both miR-615-3p and its proposed target ST3GAL2 mRNA clearly revealed anti-correlated transcript levels, whereas the cellular miR-615-3p concentration was increased by 4.3-fold ($P \leq 0.0001$) (Fig. 3A), which was accompanied by a significant 0.74-fold decrease in the ST3GAL2 mRNA level ($P \leq 0.05$) (Fig. 3B).

Reporter assays confirm ST3GAL2 as a target of miR-615-3p

To verify whether miR-615-3p directly binds to a target site within the 3′ UTR of ST3GAL2 and thereby repressively controls its post-transcriptional expression, we applied a dual luciferase reporter gene assay as described earlier [16]. Consequently, the 3′ UTR of murine ST3GAL2 was tested for binding sites of miR-615-3p using RNAhybrid [28] as previously described [3]. As shown in Fig. 3C, two interaction sites were identified. CMT 93 cells were transfected with a luciferase reporter plasmid containing a combination of both target sites [24] (Additional file 3), miR-615-3p mimic and a normalisation plasmid (pTKCluc). As shown in Fig. 3D, miR-615-3p mimics repressed luciferase activity by around 0.65-fold ($P \leq 0.05$) compared to nonsense transfected controls. The 0.65-fold decrease in luciferase activity corresponded to the decrease in ST3GAL2 transcript levels after RNAi using miR-615-3p mimics in CMT 93 (approximately 0.75-fold of nonsense control). Hence, our results revealed specific interactions between at least one of the ST3GAL2 binding sites and miR-615-3p.

ST3GAL2 protein levels in *C. jejuni* infected mice

After showing that miR-615-3p specifically binds to the ST3GAL2 target sites in vitro and that intestinal ST3GAL2 transcript levels decrease upon *C. jejuni* infection in vivo, we further analysed the protein levels of ST3GAL2 by means of immunofluorescence and Western blot analyses of colonic tissue samples derived from *C. jejuni* infected secondary abiotic IL-$10^{-/-}$ mice. As representatively shown in Fig. 4A and B and the Additional file 4, the immunofluorescent ST3GAL2 signal was much stronger in colon sections derived from non-infected as compared to *C. jejuni*-infected mice. However, the overall fluorescence signal in mouse colon sections stained with the anti-ST3GAL2 antibody was of low intensity. Furthermore, negative controls were analysed in serial sections on the same tissue area proving the specificity of detected signals (data shown in the Additional file 4). Moreover, *C. jejuni* infection of mice resulted in distinct histomorphological changes within the colon mucosa and lamina propria such as apoptosis of epithelial cells, villous blunting and irregular crypts as reported previously [29, 30].

In addition, we performed Western blot assays for ST3GAL2 using the same treated murine colonic tissue samples as for qPCR analysis. Compared to the significant decrease in ST3GAL2 expression at the mRNA level and the apparent decrease in immunofluorescence signal of the ST3GAL2 protein, we found only a slight trend towards down-regulation of ST3GAL2 at the protein level by Western blot in infected samples (not significant; Fig. 4C).

Discussions

The intestinal mucus layer can confine enteropathogens in the outer layer and exclude pathogens through intestinal peristalsis and mucus turnover to protect epithelial cells from attack by pathogens [31]. Despite the defensive system the mucus provides, enteric pathogens are able to breach this barrier by employing manifold strategies. For instance, *C. jejuni* is able to grow and thrive in the mucus layer and can negotiate this barrier by distinct motility [32]. However, the host responses triggered because of *C. jejuni* interaction with the mucosa are not yet fully understood at the molecular level. Accordingly, the mechanisms of whether and how the mucin glycoprotein pattern is modulated during intestinal *C. jejuni* infection and what this means for the interaction with the host are still largely unknown.

The gel-forming mucin MUC2 and the viscosity regulating trefoil factor TFF3 are essential for intestinal integrity. It has been reported that microbial infection can induce mucin secretion by goblet cells as a host response

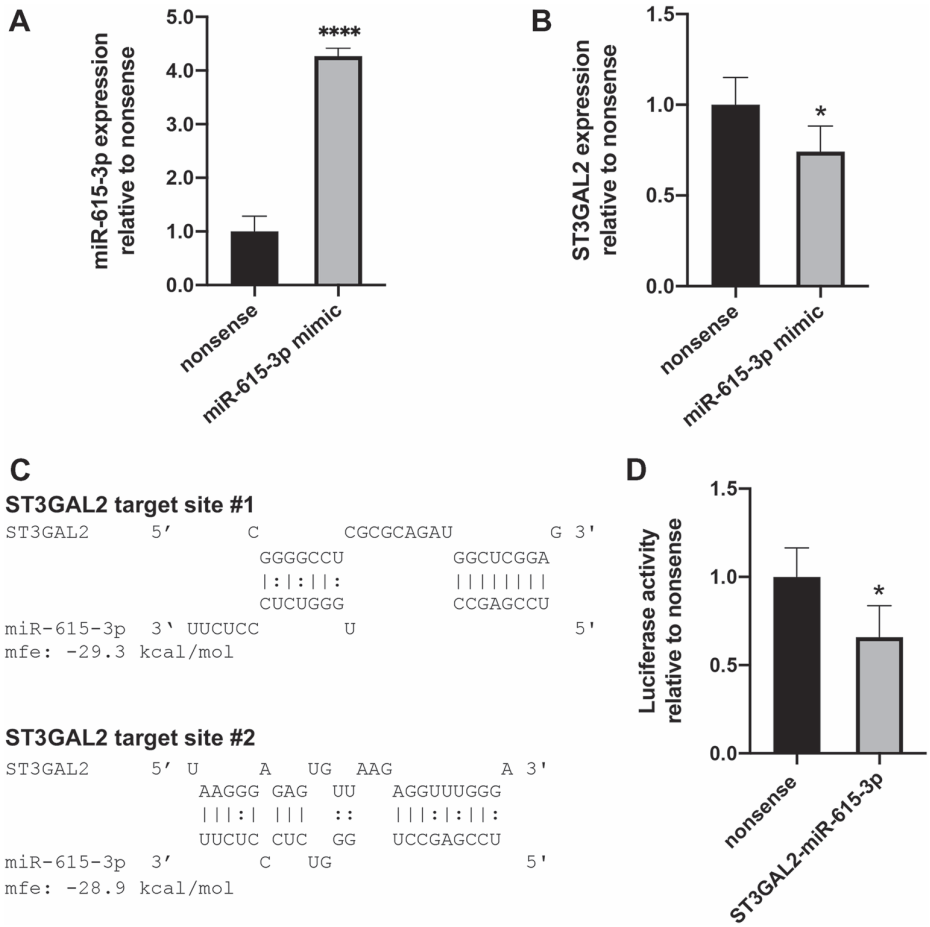

Fig. 3 Specific interaction of miR-615-3p with ST3GAL2 confirmed by RNAi and dual luciferase reporter assay. **A**, **B** RT-qPCR analysis for ST3GAL2 transcription in CMT 93 cells transfected with miR-615-3p mimics and nonsense miRNA as control. Fold change of ST3GAL2 and miR-615-3p was calculated relatively to nonsense control and normalised with HPRT, SDHA, B2M and miR-16, miR-145, respectively. Datasets are presented as means of three biological samples and double measurements ± SD. **C** Identified target sites of ST3GAL2 and miR-615-3p were analysed with RNAhybrid. **D** Relative luciferase activity was determined to the nonsense miRNA mimic serving as control. Luciferase signal was assayed at 24 h post-transfection. Dataset was plotted as average values of at least four biological replicates with standard deviation from three measurements. Columns show the mean ± SD (bars). Asterisks represent a statistical significance compared to negative controls at each time point. $^{*}P \leq 0.05$, $^{****}P \leq 0.0001$, unpaired *t*-test

to maintain and protect mucosal barrier [31]. Here, we observed significantly increased transcription of both MUC2 and TFF3 in cells derived from human colonic epithelial cell line HT-29/B6 immediately after *C. jejuni* NCTC 11168 infection. This is in agreement with a previous study, reporting increased transcriptional MUC2 expression upon *Escherichia coli* O157:H7 infection in human colon cells HT-29 [33]. In contrast to the upregulation of MUC2 and TFF3 mRNA in the initial stage of *C. jejuni* infection, we observed a slightly reduced transcription of MUC2 and profoundly decreased transcription of TFF3 in the colon of stably *C. jejuni* 81–176 infected mice (i.e., day 6 p.i.). Consistent with our observation, Bergstrom et al. found a slight reduction of MUC2 and TFF3 gene expression at 6 days p.i. and a significant decrease 10 days following murine *Citrobacter rodentium* infection [22]. According to our recent findings, *C. jejuni* strain NCTC 11168 and 81–176 exhibited comparable colonisation abilities and induced similar host responses with regard to histopathology and immune responses in secondary abiotic IL-$10^{-/-}$ mice [34, 35]. Therefore, we assume that the observed dysregulation of MUC2 and TFF3 upon *in vitro* and *in vivo C. jejuni* infection was

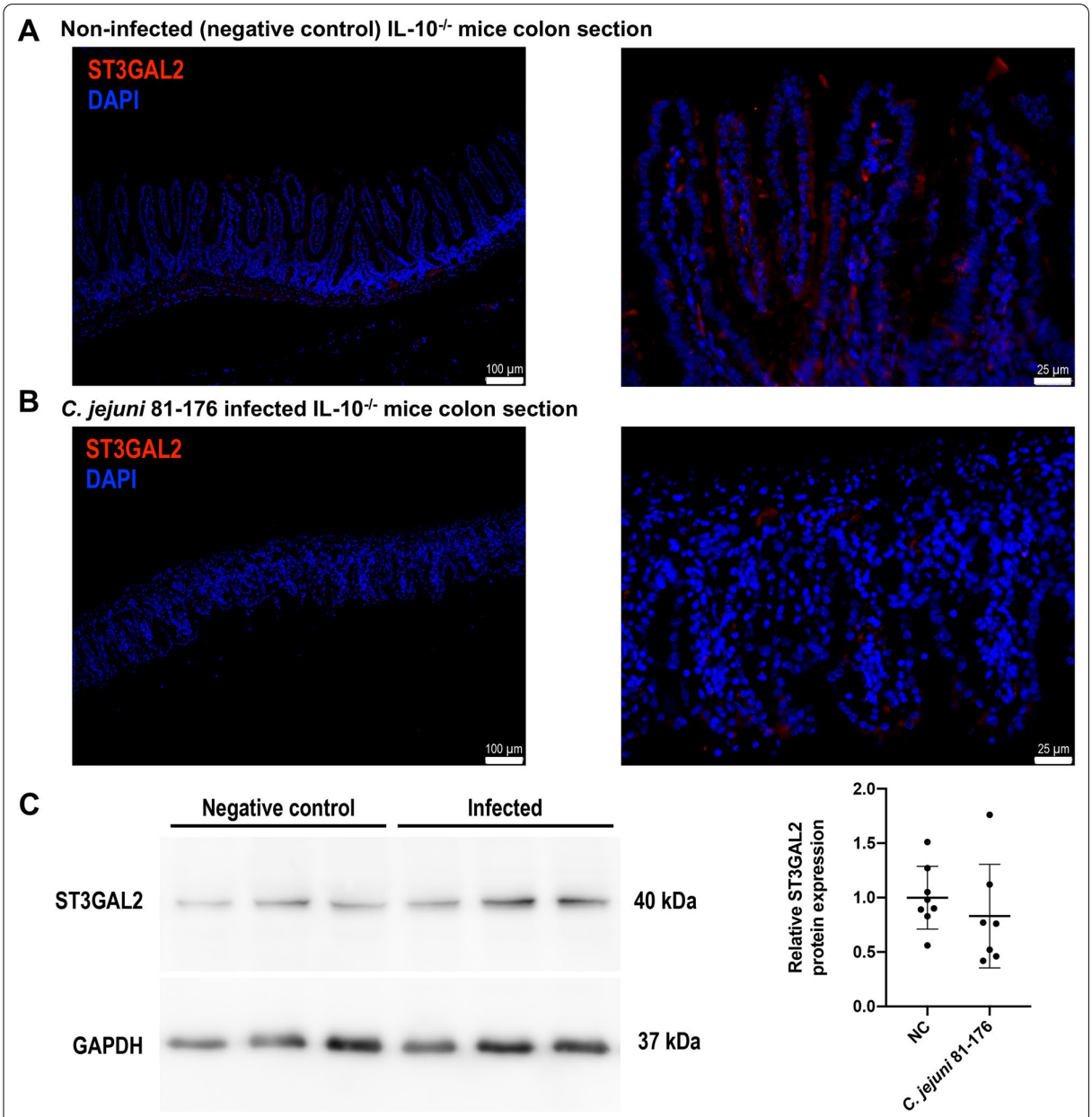

Fig. 4 Detection of protein expression of ST3GAL2 in colonic tissue of secondary abiotic IL-10$^{-/-}$ mice upon *C. jejuni* 81–176 infection. **A**, **B** Representative micrographs comparing ST3GAL2 immunofluorescence results in the colonic tissue of naïve mice (A) and *C. jejuni* 81-176-infected mice (B). ST3GAL2 was immunofluorescently labelled in red and nuclei was stained with DAPI in blue. Micrographs at left side are at magnification 10 × (Scale bar 100 µm) and right side at higher magnification 40× (scale bar 25 µm) and exposure time were the same in all sections. Data are representative for three biological replications. **C** Right panel exemplifies detection of ST3GAL2 from three individual replicates of infected and non-infected mouse colonic tissue via Western blotting. GAPDH is shown as respective loading reference. ST3GAL2 protein bands were quantified by densitometry relative to the respective GAPDH signals. Charts represent means of at least seven biological replicates ± SD, unpaired *t*-test

associated to the stage of infection rather than to the strains.

Evidence is emerging that miRNA-mediated regulation of mucins has important implications in inflammation and cancer biology [36]. For instance, miR-9 is responsible for gastric malignancies by targeting CDX2, conferring among others to enhanced proliferation of gastric cancer cells and MUC2 expression [36]. Further, miR-205 overexpression in CaCo-2 cells resulted in the accumulation of mucus-secreting goblet cell-like cells and enhanced mucin production and MUC2 expression [37]. Based on our analysis, anti-correlated expression of miR-125a-5p and miR-615-3p was associated with the dysregulation of MUC2 and TFF3 transcription at the early stage of *C. jejuni* NCTC 11168 infection *in vitro*. Likewise, we observed anti-correlated transcription of miR-615-3p with MUC2 and TFF3 mRNA upon established *C. jejuni* 81-176 infection *in vivo*. These findings suggest that the alterations of mucin expression induced by *C. jejuni* are connected with miR-125a-5p and miR-615-3p regardless of the strain. However, the direction of altered expressions seemed to be different at early and later stages of infection.

In this study, we aimed to build on the known role of MUC2 and TFF3 in intestinal bacterial infections and to identify novel regulatory aspects. For this purpose, we chose an approach that considers miRNAs as master regulators of those genes that belong to a metabolic or signalling pathway and interact accordingly. Here, we focused on the regulation of factors that are in the context of mucin modification and might be regulated by predicted miRNAs. Therefore, we concentrated our interest on target genes of miR-125a-5p and miR-615-3p, which may be related to metabolic changes of MUC2.

Upon enteric infection, quantity of host mucin may change by hypersecretion [31]. There can also be qualitative changes of mucins during infection including alterations in glycosylation. In this context, *H. pylori* was shown to change inflammation-associated mucin sialylation [31]. Previous studies have also observed altered degree of sulphation, sialylation and varied rate of glycosylation during intestinal inflammation as well as altered length and complexity of mucin glycans as a secondary effect to inflammation [38]. These alterations can further affect the adhesion of bacteria and the degradation of mucus by pathogens [38]. Thus, the interaction of mucus and pathogens seems to be a dynamic and complex process modulated by multiple factors [39]. Growing evidence has shown that miRNAs are major regulators of the glycome, playing a substantial role in modulating and controlling glycosylation [20]. However, our knowledge concerning the regulation of mucin modifying enzymes by miRNAs in bacterial infections is rather limited to date. According to our pathway analysis, several glycosyltransferases were potentially regulated by miR-125a-5p and miR-615-3p, from which we found two potential target genes (ST3GAL1, ST3GAL2) that are involved in mucin type *O*-glycan biosynthesis. *O*-glycans are the major glycans of mucins, which are generated via *O*-glycosylation and elongated or modified in a stepwise manner incorporating specific enzymes for sialylation, sulphation or acetylation [9]. ST3GAL1 and ST3GAL2 belong to a sialyltransferase family termed as ST3 β-Galactoside α-2,3-Sialyltransferase [40, 41]. ST3GAL1 predominantly adds sialic acid to the core 1 *O*-glycan [40], while ST3GAL2 transfers sialic acid to the terminal galactose residues found in glycoconjugates and it is also known for its importance in the biosynthesis of gangliosides [41]. Our RT-qPCR results showed increased ST3GAL1 mRNA expression in murine colonic tissue 6 days after *C. jejuni* infection, while a profound reduction of ST3GAL2 mRNA level was observed. This suggests that *C. jejuni* infection affects the expression of sialyltransferase genes in the mouse colon. This might occur in a miRNA-dependent manner, since predicted miRNAs (miR-125a-5p and miR-615-3p) possessed anti-correlated expression. However, whether observed miRNA-dependent alteration of sialyltransferases is only specific to *C. jejuni* infection requires more in-depth investigations. This will be the subject of our future research.

In the current study, we mainly focused on the interaction between ST3GAL2 and miR-615-3p, because on one hand, it showed typical binding properties by in silico analysis and on the other hand, our RT-qPCR analysis showed strongly negative correlation between both partners in colonic tissues derived from *C. jejuni* 81-176 infected mice. By both, the dual luciferase reporter assay and RNAi performed in CMT 93 cells, we demonstrated that miR-615-3p targets at least one predicted binding site within the 3′ UTR of the sialyltransferase ST3GAL2 specifically. Overall, the results suggest that miR-615-3p might play a regulatory role during *C. jejuni* infection of mice by targeting ST3GAL2. Nevertheless, the basic mechanism and regulatory pathway of miR-615-3p-ST3GAL2 interaction involved in sialylation upon murine *C. jejuni* infection still needs to be clarified. We also found that the transcription of ST3GAL1 and miR-125a-5p was negatively correlated. These observations could indicate a change in the mucin structure, as one sialyltransferase is down-regulated and the other up-regulated. However, whether miR-125a-5p specifically targets ST3GAL1 and how potentially altered glycosylation patterns impact the mucin structure are the questions remain to be investigated in our future studies.

In addition, we further determined the impact of *C. jejuni* 81-176 on ST3GAL2 expression at the protein level

by performing immunofluorescent staining and Western blotting. Consistent with our results showing that infection caused down-regulation of ST3GAL2 mRNA, we observed reduced staining for ST3GAL2 proteins in murine colonic tissue samples at day 6 p.i. We also quantified changes of ST3GAL2 protein level via Western blotting and found a trend towards slightly decreased levels in the mouse colon 6 days after *C. jejuni* 81-176 infection. Nevertheless, the majority of our results reveal that the expression of the sialyltransferase ST3GAL2 is regulated during infection with *C. jejuni* 81-176 by miR-615-3p. Due to the fixation, we could not follow the mucin change by staining, as the mucus was dehydrated by paraformaldehyde fixation and thus differences e.g. in thickness could not be traced [42].

Conclusions

In conclusion, using in vivo and in vitro approaches, this study demonstrated that *C. jejuni* infection induced dysregulation of the mucin MUC2 and its mediator TFF3 mRNA as well as mucin-associated miRNAs (miR-125a-5p and miR-615-3p) in an apparently strain-independent manner. Moreover, we observed aberrant transcription of sialyltransferases ST3GAL1 and ST3GAL2 that are involved in mucin type *O*-glycan biosynthesis. Our results suggest that these mucin-associated factors interact in a coordinated manner concerted by a miRNA-dependent regulatory network during *C. jejuni* infection. Herein, ST3GAL2 has been identified as a target of miR-615-3p *in vitro* and for the first time we show a regulatory relationship of miR-615-3p and ST3GAL2 involved in the host cellular response to *C. jejuni* 81-176 infection in mice. Whether the mechanisms underlying these alterations during *C. jejuni* infection involve processes that are dependent or coordinated with the regulation of other predicted miRNAs remain to be addressed. For further explorations, it could be intriguing to know the possible impact of miRNA-regulated sialyltransferases on mucin structure during *C. jejuni* infection. Moreover, we will shed light on the functional effects of miR-615-3p-ST3GAL2-interaction in response to other bacterial infections in future.

Methods

Bacterial strains, cell lines and culture conditions

Campylobacter jejuni NCTC 11168 were grown as described earlier [43]. Briefly, bacteria were routinely grown on Mueller-Hinton agar (Oxoid, Munich, Germany) supplemented with 5% defibrinated sheep blood or in Brucella broth (BD, Heidelberg, Germany) at 37 °C under microaerobic conditions (10% CO_2, 6% O_2 and 85% N_2) generated by Anoxomat (Omni Life Science, Bremen, Germany). *C. jejuni* NCTC 11168 were grown to mid-exponential phase in BB and centrifuged (14,000×*g*, 5 min), and re-suspended in the cell culture medium prior to infection assay in vitro. For in vivo infection, a stock solution of *C. jejuni* 81–176 strain (stored in −80 °C) was thawed and aliquots streaked onto Karmali agar (Oxid, Wesel, Germany) and incubated a under microaerobic conditions for 48 h, as prev described [44]. Bacteria were harvested with a final inoculum of 10^9 bacterial cells in sterile phosphate buffered saline (PBS) (Gibco, Life Technologies, UK) immediate prior to peroral infection of mice.

The subclone HT-29/B6 [45] of the human colorectal adenocarcinoma cell line HT-29 (DSMZ_ACC299) was maintained as previously described [43]. Briefly, HT-29/B6 cells were routinely cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) FCS superior (Biochrom, Berlin, Germany) in 75 cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany) at 37 °C and 5% CO_2 under a humidified atmosphere until a confluence of approx. 80% was reached. For infection assays, 5×10^5 HT-29/B6 cells were seeded into each well of a 6 well plate (Sarstedt) and incubated for 7 days with changing the media regularly. Murine rectum carcinoma cell line CMT 93 (ECACC 89111413) was cultured as described by Jonckheere et al. [46]. Briefly, CMT 93 was maintained in high glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM, Lonza, Köln, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Biochrom, Cambridge, United Kingdom). Cultivation of cells was performed in 75 cm^2 tissue culture flasks (Sarstedt, Nümbrecht, Germany) at 37 °C in a humidified 5% CO_2 atmosphere until a confluence of approx. 75% was reached. For nucleofection, 1×10^6 CMT 93 cells were taken from pre-cultured CMT 93 cells with 75% confluency, followed with transfection and 24 h incubation.

Animal experiments and tissue sampling

Mouse experiments were carried out in compliance with the European Guidelines for animal welfare (2010/63/EU). The protocol was approved by the commission for animal experiments headed by the "Landesasmt für Gesundheit und Soziales" (LaGeSo, Berlin, registration numbers G0172/16 and G0247/16). Animal welfare was monitored twice daily by assessment of clinical conditions. As previously described [30], secondary abiotic IL-$10^{-/-}$ mice (C57BL/6j background) from the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité-University Medicine Berlin) were included in the study. Infected animals (in total n = 12) were perorally challenged with 0.3 ml PBS containing 10^9 colony forming units (CFU) of *C. jejuni* strain 81-176, while control animals (in total n = 11) were challenged with 0.3

ml PBS only at day 0 and 1. Animals of both groups were matched by sex and age, respectively. Mice were strictly kept in a sterile environment to avoid contaminations. At 6 days post infection, mice were sacrificed by cervical dislocation and intestinal samples were taken under aseptic conditions following the method previously described [44]. Intestinal tissue samples were obtained in parallel from each mouse for further immunofluorescence assays. Tissue sample lysis, RNA extraction, cDNA synthesis as well as protein isolation are described below.

In vitro infection assay

Campylobacter jejuni infection using the cell line HT-29/B6 was performed as described previously [43]. Briefly, HT-29/B6 cells were infected with a suspension of approx. 1×10^9 CFU of *C. jejuni* NCTC 11168 (MOI 500). Cell monolayers were infected for 1, 5 and 24 h post infection, respectively. Gentamicin (300 ng/ml, Biochrom) protection was performed after 3 h to kill extracellular bacteria. Before harvesting, infected cells were washed with PBS 3 times and lysed directly in each well by addition of cell lysis buffer (Roboklon, Berlin, Germany) before total RNA was extracted as described below. Non-infected cells (negative control) were treated under the same conditions and harvested at same time points, respectively.

RNA extraction, cDNA synthesis and real-time qPCR analysis

Total RNA was isolated, and quality controlled as described earlier [3, 43]. Briefly, total RNA from HT-29/B6 cells was extracted by using the Universal RNA/miRNA Purification Kit (Roboklon, Berlin, Germany) according to the manufacturer's protocol. Total RNA from murine colonic tissue and CMT 93 cells was isolated with the miRVana Isolation Kit (Life Technologies, Germany). The cDNA was synthesised from individual cell or tissue samples as described earlier [47]. Basically, the isolated total RNA was treated with DNase I (RNase-Free) (NEB GmbH, Frankfurt a/M, Germany) to exclude residual genomic DNA. 1 µg total RNA was reverse transcribed using 200 U M-MuLV Reverse Transcriptase (Thermo Fisher Scientific), 0.2 µg random hexamers (Thermo Fisher Scientific), 200 µM of each dNTP and 1× supplied RT buffer. Control samples were treated in the same way but without M-MuLV Reverse Transcriptase to monitor the presence of genomic DNA. Quantification of mRNA as well as miRNA expression via RT-qPCR was performed as described previously [15]. Expression data was normalised with calculated geometric means of stably expressed reference genes determined beforehand using geNorm [48]. For normalisation of mRNA expression in HT-29/B6, ACTB and B2M were used as reference genes and for normalisation of miRNA expression the small RNAs SNORD44 along with SNORD47 were used as reference [49]. For normalisation of mRNA expression in mouse colon tissue HPRT and SDHA were used as reference and miRNA expression was normalised to the miRNAs miR-16 and miR-145-5p. HPRT, SDHA and B2M were used for normalisation of mRNA expression in CMT 93 cells and miRNA expression was normalised considering miR-16 and miR-145-5p as references. The relative gene expression was calculated by the ΔΔCt method [50] as described earlier [15]. For the cDNA synthesis and RT-qPCR analysis (miR-Q) of the miR-320-family, special RT-6 primer and reverse PCR primer (Additional file 3) with corresponding base ambiguities were generated to cover the entire members of the miR-320 family shown in Fig. 1 as described earlier [51]. Oligonucleotides used in this study were all synthesised by Sigma-Aldrich (Darmstadt, Germany) and are listed in Additional file 3.

Western blotting

Protein from colonic tissue samples of *C. jejuni* 81–176 infected secondary abiotic IL-$10^{-/-}$ mice was isolated and quantified as previously described with a few modifications [47, 52]. Briefly, tissue was lysed in cold RIPA buffer supplemented with protease inhibitor cocktail (Thermo Fisher Scientific). Protein samples were quantified using a BCA assay (Thermo Fisher Scientific). Equal amounts of protein (30 µg) were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (PVDF) (GE Healthcare, Buckinghamshire, UK) via semi-dry blotting. After blocking in 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in TBST (Tris-HCl-buffer with 0.1% (v/v) tween-20) for 2 h at room temperature, the membranes were probed with the primary antibodies (Rabbit anti-ST3GAL2, Novus Biologicals, Colorado, USA), 1:500 in 3% BSA in TBST at 4 °C overnight. Membranes were then washed three times in TBST for 15 min and subsequently incubated with horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody (1:5000 in 3% BSA in TBST; Cell Signalling Technology) for 2 h at room temperature. Immuno-reactive proteins were developed by using the Amersham™ ECL Select™ Western Blotting Detection Reagent (GE Healthcare). After detection of ST3GAL2, membrane was stripped and again processed for GAPDH detection with Rabbit anti-GAPDH (#5174; Cell Signalling Technology, Danvers, MA, USA, 1:2000 in 3% BSA in TBST) and HRP-linked anti-rabbit IgG antibody (1:5000 in 3% BSA in TBST; Cell Signalling Technology). Protein quantification was performed by densitometry using the software BIO-1D (Vilber

Lourmat, Marne-la-Vallée, France). Experiments were repeated at least five times.

Immunofluorescence

For immunofluorescence detection, mouse colon sampling and immunostaining were performed as described earlier with few modification [25, 44, 53]. Briefly, mouse colon samples were immediately fixed in 5% formalin and embedded in paraffin before serial sections were cut. 5 µm of paraffin sections were deparaffinated in Roticlear (Carl Roth, Karlsruhe, Germany) and rehydrated through a graded series of ethanol followed by rinsing in distilled water and PBS (pH 7.4, Sigma-Aldrich). Non-specific binding was blocked with 1% (v/v) BSA (Sigma-Aldrich) in PBST (0.1% (v/v) Tween-20 in PBS) for 1 h at room temperature. Thereafter, sections were incubated with a 1:50 dilution of primary antibody, Rabbit anti-ST3GAL2 (Novus Biologicals), in PBST containing 1% (v/v) BSA overnight at 4 °C. Negative controls were performed without using the primary antibody. After three washes with PBS for 5 min, the primary antibody was detected with goat anti-rabbit IgG DyLight 594 (1: 400, #35561; Thermo Fisher Scientific) for 1 h at room temperature followed by two washing steps with PBS. Nuclei were counterstained with 200 ng/ml 4′, 6-diamidin-2-phenylindol (DAPI, Sigma-Aldrich) in PBS by 3 min incubation at room temperature. Subsequently, slides were washed in PBS and mounted with Prolong™ Diamond Antifade Mountant (Life Technology). Fluorescence microscopy was performed using a Leica DMI6000B inverted microscope and the Leica LAS-X software (Leica, Wetzlar, Germany). Immunofluorescence images were taken under identical microscope and camera settings. Images were taken with background-subtraction and at least four random areas per section and more than ten images per area were selected. Experiments were carried out with three biological replicates.

RNAi and luciferase reporter assay

CMT 93 cells were cultured as described above and transfected via electroporation using the Nucleofector Technology (Lonza AG, Köln, Germany) as previously described with some modifications [3]. For nucleofection, 1×10^6 cells were used together with 50 pmol of hsa-miR-615 miRVana miRNA mimics (#4464066; Fisher Scientific, Schwerte, Germany) or 50 pmol non-targeting siRNA (D-001810-03-05, Dharmacon Lafayette, CO, USA) as control. Twenty-four hours after transfection, cells were washed with PBS and lysed for RNA isolation.

Dual luciferase reporter assays were performed according to the previous study [15]. For generation of reporter plasmids, the identified target site of murine ST3GAL2 was amplified using the hybridised oligonucleotides NotI-mmuST3GAL2-ts-sense and XbaI-mmuST3GAL2-ts-antisense obtained from Sigma-Aldrich (Additional file 3). The target site was cloned in pTK-Gluc (NEB GmbH) using the restriction enzymes NotI and XbaI (NEB GmbH). The endotoxin-free reporter plasmid (pTKGmST3GAL2) was produced for transfection using NucleoBond Xtra Midi Plus EF (Macherey-Nagel GmbH & Co. KG, Düren, Germany). CMT 93 cells were transfected using 1.3 µg of pTKGmST3GAL2 and 200 ng normalisation plasmid (pTK-Cluc, NEB GmbH) along with 150 pmol Pre-miR miRNA Precursor hsa-miR-615 (Life Technologies) or 150 pmol non-sense miRNA Pre-miR miRNA Precursor Negative Control #1 (Life Technologies) as negative control. Gaussia/Cypridina Luciferase activity was detected three times for at least four independent experiments by using the Biolux Assay Kits (NEB GmbH).

In silico-analysis

For predicting miRNAs that mutually target MUC2 and TFF3, miRmap analysis [23] was performed using a score of ≥ 50 and lists of targets were intersected using Cytoscape [54] as described earlier [24, 52]. To emphasise the biological relevance, only miRNAs were further considered that were evolutionary conserved among human, mouse and rat (Additional file 1). After evaluating anti-correlated expression between predicted miRNAs and MUC2/TFF3 in HT-29/B6, miR-125a-5p and miR-615-3p were selected for further analysis. Their targets were predicted by performing another round of miRmap analysis and lists of targets were intersected by Cytoscape (Additional file 1). KEGG pathway enrichment was performed using Cytoscape [54] and ClueGO [55]. Functionally grouped gene ontologies and pathways were determined as described previously [52] using following settings: significant KEGG pathways enrichment ($P < 0.05$), correction method: Bonferroni step down, number of genes ≥ 4, min percentage ≥ 10.0 and *Kappa* Score ≥ 0.3.

Statistical analysis

The unpaired t-test was used in this study to compare each treatment with control. All tests were conducted applying GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA, www.graphpad.com). Asterisks in figures summarise P values ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13099-021-00437-1.

Additional file 1. Raw data of in silico analysis. Sheet 1 shows the lists of miRNAs that target MUC2 and TFF3 as well as the intersection. Sheet 2 and 3 show the miRmap analysis of the mmu-miR-615-3p and mmu-miR-125a-5p targets. Sheet 4 shows the intersection of targets.

Additional file 2. Gene enrichment analysis by means of ClueGO analysis.

Additional file 3. Oligonucleotides used in this study. Sheet 1 shows the oligonucleotide sequences of mRNAs and sheet 2 shows the oligonucleotide sequences of the selected region of ST3GAL2. Sheet 3 shows the oligonucleotide sequences of miRNAs.

Additional file 4. Immunofluorescence detection of ST3GAL2 in colonic tissue of secondary abiotic IL-10$^{-/-}$ mice upon *C. jejuni* 81–176 infection. (a, b) ST3GAL2 immunofluorescence in colonic tissue of non-infected mice out of two individual experiments (left panels: scale bar 100 µm, right panels: scale bar 25 µm). (c, d) Immunostaining of ST3GAL2 performed twice individually in colonic tissue of *C. jejuni* 81–176 infected mice (left panels: scale bar 100 µm, right panels: scale bar 25 µm). (e) Negative controls in serial sections on the same tissue area shown in Fig. 4A (left), supplementary Fig. a (middle) and Fig. b (right) (scale bar 25 µm). (f) Negative controls in serial sections for the same tissue area shown in Fig. 4B and supplementary Fig. c & d, respectively, (scale bar 25 µm). ST3GAL2 was immunofluorescently labelled in red and nuclei was stained with DAPI in blue.

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Authors' contributions
DX: Performed experiments, analysed data, wrote paper. LH: Performed experiments, analysed data. TA, RE, SB: Provided advice in study design, co-edited paper. MMH, GG, SS: Designed study, performed experiments, analysed data, co-wrote paper. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Mouse experiments were carried out in compliance with the European Guidelines for animal welfare (2010/63/EU). The protocol was approved by the commission for animal experiments headed by the "Landesasmt für Gesundheit und Soziales" (LaGeSo, Berlin, registration numbers G0172/16 and G0247/16). Animal welfare was monitored twice daily by assessment of clinical conditions.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

1. Hansson GC. Role of mucus layers in gut infection and inflammation. Curr Opin Microbiol. 2012;15(1):57–62.
2. Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A. 2011;108(Suppl 1):4659–65.
3. Hanisch C, Sharbati J, Kutz-Lohroff B, Huber O, Einspanier R, Sharbati S. TFF3-dependent resistance of human colorectal adenocarcinoma cells HT-29/B6 to apoptosis is mediated by miR-491-5p regulation of lncRNA PRINS. Cell Death Discov. 2017;3:16106.
4. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep. 2010;12(5):319–30.
5. Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A. 2008;105(39):15064–9.
6. Mashimo H, Wu DC, Podolsky DK, Fishman MC. Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. Science. 1996;274(5285):262–5.
7. Stone EL, Lee SH, Ismail MN, Fukuda M. Characterization of mice with targeted deletion of the gene encoding core 2 beta1,6-N-acetylglucosaminyltransferase-2. Methods Enzymol 2010, 479:155–72.
8. Poole J, Day CJ, von Itzstein M, Paton JC, Jennings MP. Glycointeractions in bacterial pathogenesis. Nat Rev Microbiol. 2018;16(7):440–52.
9. Jensen PH, Kolarich D, Packer NH. Mucin-type O-glycosylation–putting the pieces together. FEBS J. 2010;277(1):81–94.
10. Sicard JF, Le Bihan G, Vogeleer P, Jacques M, Harel J. Interactions of Intestinal Bacteria with Components of the Intestinal Mucus. Front Cell Infect Microbiol. 2017;7:387.
11. Naughton JA, Marino K, Dolan B, Reid C, Gough R, Gallagher ME, Kilcoyne M, Gerlach JQ, Joshi L, Rudd P, et al. Divergent mechanisms of interaction of Helicobacter pylori and Campylobacter jejuni with mucus and mucins. Infect Immun. 2013;81(8):2838–50.
12. Silva J, Leite D, Fernandes M, Mena C, Gibbs PA, Teixeira P. Campylobacter spp. as a Foodborne Pathogen: A Review. Front Microbiol. 2011;2:200.
13. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol. 2019;20(1):21–37.
14. Eulalio A, Schulte L, Vogel J. The mammalian microRNA response to bacterial infections. RNA Biol. 2012;9(6):742–50.
15. Sharbati J, Lewin A, Kutz-Lohroff B, Kamal E, Einspanier R, Sharbati S. Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection. PLoS One. 2011;6(5):e20258.
16. Hoeke L, Sharbati J, Pawar K, Keller A, Einspanier R, Sharbati S. Intestinal Salmonella typhimurium infection leads to miR-29a induced caveolin 2 regulation. PLoS One. 2013;8(6):e67300.
17. Sun YM, Lin KY, Chen YQ. Diverse functions of miR-125 family in different cell contexts. J Hematol Oncol. 2013;6:6.
18. Zhou X, Chen H, Zhu L, Hao B, Zhang W, Hua J, Gu H, Jin W, Zhang G: Helicobacter pylori infection related long noncoding RNA (lncRNA) AF147447 inhibits gastric cancer proliferation and invasion by targeting MUC2 and up-regulating miR-34c. Oncotarget. 2016, 7(50):82770–82.
19. Singh N, Shirdel EA, Waldron L, Zhang RH, Jurisica I, Comelli EM. The murine caecal microRNA signature depends on the presence of the endogenous microbiota. Int J Biol Sci. 2012;8(2):171–86.
20. Kasper BT, Koppolu S, Mahal LK. Insights into miRNA regulation of the human glycome. Biochem Biophys Res Commun. 2014;445(4):774–9.
21. Kurcon T, Liu Z, Paradkar AV, Vaiana CA, Koppolu S, Agrawal P, Mahal LK. miRNA proxy approach reveals hidden functions of glycosylation. Proc Natl Acad Sci USA. 2015;112(23):7327–32.

22. Bergstrom KS, Guttman JA, Rumi M, Ma C, Bouzari S, Khan MA, Gibson DL, Vogl AW, Vallance BA. Modulation of intestinal goblet cell function during infection by an attaching and effacing bacterial pathogen. Infect Immun. 2008;76(2):796–811.
23. Vejnar CE, Zdobnov EM. MiRmap: comprehensive prediction of microRNA target repression strength. Nucleic Acids Res. 2012;40(22):11673–83.
24. Pawar K, Sharbati J, Einspanier R, Sharbati S. *Mycobacterium bovis* BCG interferes with miR-3619-5p control of cathepsin S in the process of autophagy. Front Cell Infect Microbiol. 2016;6:27.
25. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kuhl AA, Dasti JI, Zautner AE, Munoz M, Loddenkemper C, et al. Novel murine infection models provide deep insights into the "menage a trois" of Campylobacter jejuni, microbiota and host innate immunity. PLoS ONE. 2011;6(6):e20953.
26. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27–30.
27. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: database for annotation, visualization, and integrated discovery. Genome Biol. 2003;4(5):P3.
28. Kruger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. Nucleic Acids Res. 2006, 34(Web Server issue):W451–454.
29. Erben U, Loddenkemper C, Doerfel K, Spieckermann S, Haller D, Heimesaat MM, Zeitz M, Siegmund B, Kuhl AA. A guide to histomorphological evaluation of intestinal inflammation in mouse models. Int J Clin Exp Pathol. 2014;7(8):4557–76.
30. Haag LM, Fischer A, Otto B, Plickert R, Kuhl AA, Gobel UB, Bereswill S, Heimesaat MM. *Campylobacter jejuni* induces acute enterocolitis in gnotobiotic IL-10-/- mice via Toll-like-receptor-2 and – 4 signaling. PLoS One. 2012;7(7):e40761.
31. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. Mucosal Immunol. 2008;1(3):183–97.
32. Alemka A, Corcionivoschi N, Bourke B. Defense and adaptation: the complex inter-relationship between Campylobacter jejuni and mucus. Front Cell Infect Microbiol. 2012;2:15.
33. Xue Y, Zhang H, Wang H, Hu J, Du M, Zhu MJ. Host inflammatory response inhibits Escherichia coli O157:H7 adhesion to gut epithelium through augmentation of mucin expression. Infect Immun. 2014;82(5):1921–30.
34. Bereswill S, Mousavi S, Weschka D, Buczkowski A, Schmidt S, Heimesaat MM. Peroral clove essential oil treatment ameliorates acute Campylobacteriosis-results from a preclinical murine intervention study. Microorganisms. 2021, 9(4):735
35. Schmidt AM, Escher U, Mousavi S, Boehm M, Backert S, Bereswill S, Heimesaat MM. Protease activity of *Campylobacter jejuni* HtrA modulates distinct intestinal and systemic immune responses in infected secondary abiotic IL-10 deficient mice. Front Cell Infect Microbiol. 2019;9:79.
36. Krishn SR, Batra SK, Kaur S. Advances in miRNA-mediated mucin regulation. Curr Pharmacol Rep. 2015;1(6):355–64.
37. Eyking A, Reis H, Frank M, Gerken G, Schmid KW, Cario E. MiR-205 and MiR-373 are associated with aggressive human mucinous colorectal cancer. PLoS ONE. 2016;11(6):e0156871.
38. Kudelka MR, Stowell SR, Cummings RD, Neish AS. Intestinal epithelial glycosylation in homeostasis and gut microbiota interactions in IBD. Nat Rev Gastroenterol Hepatol. 2020;17(10):597–617.
39. McGuckin MA, Linden SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. Nat Rev Microbiol. 2011;9(4):265–78.
40. Angata K, Fukuda M: ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 1 (ST3GAL1). In: Taniguchi N, Honke K, Fukuda M, Narimatsu H, Yamaguchi Y, Angata T Eds. Handbook of Glycosyltransferases and Related Genes. Tokyo: Springer Japan; 2014, 637–644.
41. Tsuji S, Takashima S: ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 2 (ST3GAL2). In: Taniguchi N, Honke K, Fukuda M, Narimatsu H, Yamaguchi Y, Angata T Eds. Handbook of Glycosyltransferases and Related Genes. Tokyo: Springer Japan; 2014, 645–656.
42. Hansson GC, Johansson ME. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Gut Microbes. 2010;1(1):51–4.
43. Xi, Alter T, Einspanier R, Sharbati S, Golz G. Campylobacter jejuni genes Cj1492c and Cj1507c are involved in host cell adhesion and invasion. Gut Pathog. 2020;12:8.
44. Mousavi S, Escher U, Thunhorst E, Kittler S, Kehrenberg C, Bereswill S, Heimesaat MM. Vitamin C alleviates acute enterocolitis in *Campylobacter jejuni* infected mice. Sci Rep. 2020;10(1):2921.
45. Kreusel KM, Fromm M, Schulzke JD, Hegel U. Cl- secretion in epithelial monolayers of mucus-forming human colon cells (HT-29/B6). Am J Physiol. 1991;261(4 Pt 1):C574–82.
46. Jonckheere N, Van Der Sluis M, Velghe A, Buisine MP, Sutmuller M, Ducourouble MP, Pigny P, Buller HA, Aubert JP, Einerhand AW, et al. Transcriptional activation of the murine Muc5ac mucin gene in epithelial cancer cells by TGF-beta/Smad4 signalling pathway is potentiated by Sp1. Biochem J. 2004;377(Pt 3):797–808.
47. Scholven J, Taras D, Sharbati S, Schon J, Gabler C, Huber O, Meyer zum Buschenfelde D, Blin N, Einspanier R. Intestinal expression of TFF and related genes during postnatal development in a piglet probiotic trial. Cell Physiol Biochem. 2009;23(1–3):143–56.
48. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034.
49. Sharbati S, Sharbati J, Hoeke L, Bohmer M, Einspanier R. Quantification and accurate normalisation of small RNAs through new custom RT-qPCR arrays demonstrates Salmonella-induced microRNAs in human monocytes. BMC Genom. 2012;13:23.
50. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402–8.
51. Sharbati-Tehrani S, Kutz-Lohroff B, Bergbauer R, Scholven J, Einspanier R. miR-Q: a novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample. BMC Mol Biol. 2008;9:34.
52. Pawar K, Hanisch C, Palma Vera SE, Einspanier R, Sharbati S. Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy. Sci Rep. 2016;6:19416.
53. Sharbati J, Bohmer M, Bohmer N, Keller A, Backes C, Franke A, Steinberg P, Zeljenkova D, Einspanier R. Transcriptomic analysis of intestinal tissues from Two 90-Day feeding studies in rats using genetically modified MON810 maize varieties. Front Genet. 2017;8:222.
54. Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campilo I, Creech M, Gross B, et al. Integration of biological networks and gene expression data using Cytoscape. Nat Protoc. 2007;2(10):2366–82.
55. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pages F, Trajanoski Z, Galon J. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 2009;25(8):1091–3.

Publisher's Note

5. Summarising discussion

Numerous *in vivo* and *in vitro* studies demonstrated that *C. jejuni* encodes various virulence determinants involved in prominent disease-associated processes including bacterial adhesion to, transmigration across, invasion into and intracellular survival within infected intestinal epithelial cells (Backert et al. 2013). The present study has identified two so far poorly characterised genes being involved in bacterial adherence to and entrance into human epithelial cells. Mutation in these two genes resulted in different interaction behaviors of *C. jejuni* with host cells. To cope with bacterial intruding, the mucus layer as the first defensive barrier possesses a complex nature and offers multiple levels of protection for host cells (Linden et al. 2008). This work indicated that during *C. jejuni* infection, MUC2 mucin, the major component of the colonic mucus layer, was subjected to significant changes in gene expression and posttranscriptional modification. In addition, this modification of mucin MUC2 was found to be associated with miRNAs and glycosyltransferases in this context.

5.1. Mutation of gene *cj1492c* and *cj1507c* impacts various virulence determinants of *Campylobacter jejuni*

C. jejuni infection in human is believed to be a multifactorial process including intestinium colonisation, specific binding with the proteins on the surface of cells lining the intestinal epithelium and subsequent invasion into the intestinal cells as well as transcellular or paracellular translocation (Kreling et al. 2020). The interaction of *C. jejuni* with intestinal mucosa consequently causes damage to the intestinal barrier initiating inflammation and immune responses (Kreling et al. 2020). Mechanisms contributing to bacterial virulence have been identified, including motility, adhesion, invasion, and the intracellular survival, etc., of which adhesion and invasion are recognised as important features in *C. jejuni* pathogenesis (Javed et al. 2010; Aguilar et al. 2014). Various bacterial factors ranging from single monomeric adhesins/invasins to complex multimeric macromolecules such as secretion systems have been characterised to contribute to the adhesion and invasion of *C. jejuni* (Pizarro-Cerda and Cossart 2006; Koolman et al. 2016). Mutational studies have been used to assess the gene function of *C. jejuni*, through which a variety of different proteins of *C. jejuni* have been shown to play a role in the adhesion and invasion process (Rubinchik, Seddon, and Karlyshev 2012; Backert et al. 2013). However, there is a lack of consistency in results from different studies, as *C. jejuni* mutants exhibited various degrees of deficiency in their ability to attach and enter different host cells. Verily, due to the contradictory data and the discrepancy of experimental conditions within different studies, a consensus seems to be yet reached to the adhesion and internalisation of *C. jejuni* among researchers. Moreover, whether there are more bacterial factors that directly or indirectly affect the interaction of *C. jejuni* with host cells, warrant further

study. The first part of this study aimed to identify further genes that may play a role in bacterial adhesion and invasion. To achieve this, the ability of individual transposon inserted mutant of *C. jejuni* NCTC 11168 to adhere to and invade into human intestinal epithelial cells was evaluated compared to the parental strain.

The mutant of *C. jejuni* with transposon located within gene *cj1492c* possessed a profoundly increased adhesive and invasive properties to HT-29/B6 cells. Gene *cj1492c* is annotated as a two-component sensor belonging to the two-component regulatory system (TCS) (Parkhill et al. 2000). TCSs can activate and/or repress transcription of different sets of genes and thus are used by many pathogens to link environmental stimulation with transcription of specific genes (Luethy et al. 2015). Compared to other bacterial species, *C. jejuni* contains only seven cognate TCSs that are encoded in the genome. Among them, FlgSR, DccRS, PhosSR, CprRS, and RacRS are five cognate TCSs have been characterised to imply controlling genes involved in host interaction, whereas PhosSR TCS proved not to be required for *in vivo* colonisation of *C. jejuni* (Luethy et al. 2015). The other cognate TCS is encoded by gene *Cjj1484* (the orthologue of Cj1492c) and gene *Cjj1483* in *C. jejuni* 81-176. This TCS seems to be conserved in many *C. jejuni* strains. It plays a role in the transcriptional repression of genes involved in commensal colonisation of chicks, metabolic processes related to iron/heme acquisition and the respiration required for *C. jejuni* growth *in vitro* (Luethy et al. 2015). Homology searches using BLASTP showed that Cj1492c is identical to Per-Arnt-Sim motif (PAS) domain-containing sensor histidine kinase. The PAS domain-containing sensor histidine kinase has not been well described in *C. jejuni*, while it has been shown to adapt responses and sense environment in pathogenic bacteria *Treponema denticola* (Sarkar et al. 2018). Moreover, another study on *Xanthomonas campestris* pv. *campestris* has reported that RpfS, a complex sensor kinase containing PAS domains, is involved in sensing multiple environmental or cellular signals (An et al. 2014). Work form An et al. found that mutation of Rpfs influences motility and virulence of *Xanthomonas campestris* pv. *campestris* (An et al. 2014). In the current study, mutant ::Cj1492c exhibited profoundly enhanced adhesion and invasion ability (approx. 3-fold more than that of the wild type), while one-half of survival rate in human epithelial cells HT-29/B6 compared to the wild type. Moreover, the mutagenesis of gene *cj1492c* resulted in a decreased growth rate compared to the wild type and a slightly reduced swarming ability (73% of that of wild type).

Another mutated gene investigated in this study, *cj1507c*, is described as a regulatory protein. BLASTP search revealed that homologues of Cj1507c are LysR family transcriptional regulator (LTTR) suggesting a possible regulatory function of this protein. LTTR consists of several individual regulatory proteins that play a role in controlling biological processes involved in

bacterial survival and environmental adaptation (Schell 1993; Maddocks and Oyston 2008). Another LTTR of *C. jejuni* is Cj1000. Work from Dufour et al. shown that the *cj1000* mutant exhibited severely attenuated ability in colonisation *in vivo*. This result indicated that Cj1000 has implications in regulation of adaption pathway and thereby plays a role in host colonisation and respiration (Dufour et al. 2013). Moreover, the LysR-type regulator (RovM) in *Y. pseudotuberculosis* is proposed as a candidate to control motility, invasion and virulence of *Y. pseudotuberculosis* (Heroven and Dersch 2006). As Batisson et al. reported, the Paa protein (contains substrate binding domain of LTTR) in *E. coli* may play a transport or other functional role that indirectly affects the expression of adhesins (Batisson et al. 2003). In addition, Cj1507c exhibited substantial identity to LrhA from *E. coli* (54.55% identity) which play a vital role in regulating motility and biofilm formation (Lehnen et al. 2002). Results in the current study shown that mutation in gene *cj1507c* resulted severely reduced adherence and invasiveness of *C. jejuni* to HT-29/B6 cells. Moreover, mutant ::Cj1507c possessed the impaired capability to internalise into HT-29/B6 cells (27% of that of the wild type) and shown a significantly reduced intracellular survival rate (one-tenth of the wild type).

Noteworthy is that mutants ::Cj1492c and mutant ::Cj1507c possess the ability of adhesion and invasion in a reverse manner, however, retain similar motility. Both of them are slightly reduced (around 80%) compared to the wild type. Previous evidence has shown that the purified flagella specifically adhered to INT-407 cells indicating that immobilisation of flagella can facilitate adhesion of *C. jejuni* to host cells. Therefore, motility was considered to seriously interfere with the attaching process. Furthermore, flagellar mediated motility is suggested to be required for the attachment and the colonisation of *C. jejuni* (Rubinchik, Seddon, and Karlyshev 2012). Mutation of *flaA* resulted in significantly diminished motility and reduced adhesion and invasion to the human intestinal epithelial cells (Bolton 2015; van Vliet and Ketley 2001; Young, Davis, and Dirita 2007). Experimental evidence also shown that *C. jejuni* mutants with severely diminished invasion capability are negatively affected in their motility (Backert and Hofreuter 2013; Cróinín and Backert 2012). Nonetheless, there are other studies demonstrated that motility is necessary but not substantial for the adhesion and invasion processes, as there were clinical isolates that exhibited intact motility but significantly reduced adhesion and invasion capability (Backert and Hofreuter 2013). In addition, work from Goon et al. shown that the mutation in gene *cj0997* resulted in no defect in motility but impaired invasion, while Novik et al. demonstrated that their cj0997 mutant exhibited a reduced motility and concomitant invasion defect (Goon et al. 2006; Novik, Hofreuter, and Galan 2010). These findings collectively may indicate that the biological property of *C. jejuni* like motility is not virulence *per se* but constitute a critical requirement for colonisation and internalisation.

Moreover, genes involved in intracellular survival seem to be also associated with invasion (Backert and Hofreuter 2013; Novik, Hofreuter, and Galán 2010). Intracellular survival is a typical feature of *C. jejuni*, however, *C. jejuni* intracellular fate is still not fully understood. *C. jejuni* intercellular survival has been reported to be strongly influenced by the nature of CCV where *C. jejuni* resides after the internalisation into the epithelial cells (Bouwman, Niewold, and van Putten 2013; Watson and Galan 2008). In addition, genes involved in processes like oxidative stress, synthesis of polysaccharides (LOS, CPS), glycosylation and maintenance of metabolic activity in the internalised state are believed to have predominant impacts on the survival of internalised *C. jejuni* (Kovacs et al. 2020). In this study, mutant ::Cj1492c and mutant ::Cj1507c both exhibited impaired intracellular survival, while mutant ::Cj1507c with deficient adhesion and invasion exhibited much lower intracellular survival. It has been reported that *C. jejuni* undergoes intracellular metabolic reprogramming upon infection, which may increase the difficulty for *C. jejuni* to survive in cell (Pryjma et al. 2012; Watson and Galan 2008). Therefore, it was assumed that the mutagenesis of gene *cj1492c* and *cj1507c* might cause an impaired physiological adaptation of *C. jejuni* during the environmental variation, thereby leading to a reduced intracellular survival.

Furthermore, given that bacterial adhesion is positively correlated with host entry events (Backert et al. 2013), the invasive potential of each mutant was evaluated by the ratio of the number of internalised bacteria and the number of adherent bacteria in this study (unpublished data). The results indicated that 25% of the adherent wild type cells was internalised at 5 hour post infection, while only 5% of the adherent mutant ::Cj1507c invaded HT-29/B6 cells. In contrast, the invasive potential of mutant ::Cj1492c is 34%, which is approximately 1.5-fold elevated bacterial load compared to the wild type. Interestingly, the invasive potential of strains determined in this study is consistent with their adhesion ability, which is supported by the previous studies indicating that adherence is a critical requirement prior to the entry process (van Vliet and Ketley 2001). Otherwise, there is the presence of multiple invasion-dependent factors in addition to adherence. Work from Golden et al. has shown that mutants with an increased adherence failed to deliver a higher invasion level (Golden and Acheson 2002). Additionally, it has been observed in this study that the mutagenesis of Cj1492c and Cj1507c did not impact the induction of IL-8 expression and morphological changes in host cells. As the wild type did not impair host cell viability in the time course of our experiments, which might be due to the short period of infection, we cannot exclude potential effects of mutations on host cell cytotoxicity upon infection at later time points. The altered invasiveness of both mutants seems not to be correlated with cytotoxicity in this context.

In conclusion, we identified two mutants of *C. jejuni* possessing altered phenotypes with transposon insertions in genes *cj1492c* and *cj1507c*. These two genes are predicted to encode proteins related to TCS and LTTR. To our knowledge, no relevant report suggests that these proteins are proposed to be directly involved in the adherence and entry processes to date. Based on the results obtained in this study, genes regulated by Cj1507c have an impact on efficient adhesion, invasion and intracellular survival of *C. jejuni* in HT-29/B6 cells. Signal sensing mediated by Cj1492c may function to limit attachment and internalisation, while may promote the persistence of *C. jejuni* in HT-29/B6 cells. The gene *cj1492c* and *cj1507c* could play a positive role in the physiological adaptation of *C. jejuni* to the hostile intracellular environment. The alteration in adhesion and invasion observed in these mutants may be due to the role of Cj1492c and Cj1507c in the attachment and entry process. Cj1492c and Cj1507c may function by modulating physiological adaptation of *C. jejuni* during environmental variation and/or regulating the entry processes through collaborating with other virulence determinants of *C. jejuni*. Nonetheless, these phenotypical changes are not supported by complementation yet and still lack of firm conclusion of protein function. Whether the observed phenotypes of mutants arise from polar effects generated by mutagenesis of Cj1492c and Cj1507c still remains unclear.

5.2. *Campylobacter jejuni* infection causes mucin regulation and posttranscriptional modification via a miRNA-mediated regulatory network

Despite the mucus layer provides protection in several ways, pathogens have adapted mechanisms to interact with mucins and modulate mucin synthesis and secretion, leading less effective mucus defence and enhancing their pathogenesis (Sicard et al. 2017). The interaction of *C. jejuni* and mucins elicits host response that has evoked a great interest in the past decades, but the mechanisms of mucin glycosylation during intestinal *C. jejuni* infection is still largely unknown.

Evidence have shown that pathogen infection cause mucin alteration by virtue of acting on mucin secreting cells, changing or inhibiting mucin production and modification. For instance, *Shigella flexn*eri is able to interfere with mucin secretion by attaching and accumulation on the cell surface and further reshape mucin structures by remodelling their glycosylation pattern (Sperandio et al. 2013). *Clostridium difficile* can cause dysfunction of mucus barrier and bring about major mucosal damage by producing toxin ToxA (Branka et al. 1997). In addition, mucin degradation and solubilisation resulted by *Yersinia enterocolitica* infection has been found in rabbit small intestine (Sicard et al. 2017). In this study, a significantly increased expression of both MUC2 and TFF3 at transcriptional level (around 2-fold change) was observed right after

C. jejuni infection in the human cell line HT-29/B6. Besides, a slight but significant reduced expression of MUC2 (0.80-fold change) and profoundly decreased expression of TFF3 (0.38-fold change) were found in the colon of stably *C. jejuni* infected mice at 6-day post infection. These results are in accordance with previous studies indicating that MUC2 and TFF3 expression undergo changes during intestinal diseases caused by pathogenic bacteria (Bergstrom et al. 2008; Xue et al. 2014).

Microbial infections activate host defences and induce hypersecretion of mucin, as a response of host to pathological stimuli, to maintain the mucus layer (Linden et al. 2008). This stimulation can occur directly on mucin secreting cells and/or by proinflammatory cytokine production (Sicard et al. 2017). During pathogen infection, the secretion of proinflammatory cytokines as the first warning signs for immune system also plays a significant role on the expression of gel-forming mucins (Zheng et al. 2008; Linden et al. 2008; Xue et al. 2014). MUC2 expression was proven to be stimulated by proinflammatory cytokines including IL-1, IL-6, IL-8 and TNF-α in human intestinal cancer cell lines (Iwashita et al. 2003; Enss et al. 2000; Xue et al. 2014). Interestingly, the expression pattern of IL-8 observed upon *C. jejuni* infection within HT-29/B6 cells matches the expression of both MUC2 and TFF3 in HT-29/B6 cells following the *C. jejuni* infection *in vitro*. Briefly, C. jejuni infection induced a significantly enhanced transcript level of both IL-8 and MUC2/TFF3 in HT-29/B6 cells at the initial stage and afterwards their transcriptional expression decreased to the basal level in comparison with control group. Whether there is a link between proinflammatory cytokines and mucins regulation during *C. jejuni* infection remains to be elucidated. Moreover, goblet cells are known to exert their protective role in the intestine by synthesising and secreting many mediators including MUC2 and TFF3 (Bergstrom et al. 2008). Previous studies suggest that *C. jejuni* infection results in the depletion or loss of goblet cells (Prescott et al. 1981; Bergstrom et al. 2008; Cole et al. 2006). In the study of *Citrobacter rodentium*, the reduced transcriptional expression of goblet cell-specific genes MUC2 and TFF3 was attributed to the profoundly impaired size of the goblet cell population in colon during *Citrobacter rodentium*-induced colitis (Bergstrom et al. 2008). It can be speculated that the mucus layer could be destroyed by pathogens during the course of infection followed by a stronger bacterial colonisation, leading to a reduction in the number of goblet cells and a lower expression of goblet cell-derived proteins. Nevertheless, whether the downregulation of MUC2 and TFF3 observed in mouse colon in response to the *C. jejuni* infection was due to the alteration of goblet cells, warrant further study.

There is another evidence showing that LPS on the cell membrane of bacteria can increase the production of IL-8 by goblet cells, prompting the secretion of mucin (Sicard et al. 2017). Interestingly, the glycan portion of LOS of *C. jejuni* serves function in adherence to epithelial

cells by directly interacting with glycans that are present on the host cell surface (Poole et al. 2018). The abundant *O*-glycans existing on the cell protein surface of both host and bacteria form a critical interface that profoundly influence the host-pathogen interaction. The *O*-glycans on mucin proteins can be used as nutrient for bacteria as well as act as ligands for bacterial adhesins initiating intestinium colonisation. On the other hand, a variety of bacteria possess mucus-binding protein facilitating their adherence to mucus (Sicard et al. 2017). It has been reported that the major outer membrane protein (MOMP) of *C. jejuni* serve specific function in interaction with MUC2 in intestine (Tu, McGuckin, and Mendz 2008). *C. jejuni* possesses a wide range of binding specificity for host glycans, greatly influencing the interaction with mucins. The binding of *C. jejuni* to different sugar molecule on host mucins may change as the infection stage varies (Poole et al. 2018). The specific glycan-glycan binding is considered as an important mechanism utilised by *C. jejuni* to mediate adhesion, invasion and immune evasion, etc. (Poole et al. 2018).

O-glycans are the major glycans present in mucin, which are synthesised via *O*-glycosylation and elongated or modified in a stepwise manner incorporating specific enzymes for sialylation, sulphation or acetylation (Jensen, Kolarich, and Packer 2010; Van den Steen et al. 1998). Emerging evidence have shown the importance of glycosyltransferases in *O*-glycan biosynthesis. Glycosyltransferases play a determinant role in *O*-glycan core structures and backbone extension. Moreover, the specificity of glycosyltransferases controls the nature of glycan-chain synthesis (Corfield 2018). The absence of individual glycosyltransferases may cause aberrant glycan structure with, e.g., less extension or variated modification pattern (Corfield 2018). For example, the mice deficient in glycosyltransferase GCNT2 had a reduction in core 2 *O*-glycans and an elongated *O*-mannose structure, leading an overall altered mucin composition and a defected mucus barrier (Stone et al. 2010). In addition, previous study found that the mice lacking glycosyltransferase C1GAL1 was failed to synthesise core 1 derived *O*-glycan, resulting in spontaneous colitis and reduced mucus layer (Fu et al. 2011). The altered glycosylation in mucin seems to be pertinent to various disease progression. As previously reported, impaired *O*-glycosylation on the core structures of mucin promotes the susceptibility of mice to the development of colitis (Larsson et al. 2011). Moreover, aberrant mucin glycosylation has been reported to be associated with bacterial infection (Larsson et al. 2011).

In this study, two sialyltransferases ST3GAL1 and ST3GAL2 as well as two β1,6-N-acetyl-glucosaminyltransferase GCNT1 and GCNT4 (unpublished data) were profoundly dysregulated at transcriptional level upon *C. jejuni* infection. All of these four glycosyltransferases are involved in mucin type *O*-glycan biosynthesis. More specifically, RT-qPCR data shown a significantly increased ST3GAL1 expression (3.65-fold change compared

with control) and GCNT4 expression (4.03-fold change compared with control), while a profound reduction in expression of ST3GAL2 (0.32-fold change compared with control) and GCNT1 (0.49-fold change compared to control) in the mouse colon after 6 days of *C. jejuni* infection. The data may indicate that *C. jejuni* infection strongly affects the expression of glycosyltransferase genes in a distinct different way in the mouse colon. MUC2 are highly characterised by their glycosylation pattern, which is determined by the action of glycosyltransferases (Larsson et al. 2011). Coupled with previous reports, the shifted glycosylation pattern of MUC2 with smaller and less complex glycans was associated with the development of inflammation and disease course in ulcerative colitis patients (Larsson et al. 2011). It can be speculated that the observed dysregulation of ST3GAL1, ST3GAL2 and GCNT1, GCNT4 upon *C. jejuni* infection might remodel the glycosylation pattern during MUC2 synthesis, causing different glycan profile of MUC2. Nevertheless, the possible impact of the alteration in glycosyltransferases on mucin structure and/or composition during *C. jejuni* infection are required to elucidate in more detail.

Previous studies have established the regulation of host responses by miRNA upon bacterial infection as a common phenomenon (Staedel and Darfeuille 2013). In the last decade, miRNAs have emerged as the important regulator for mucin expression, strongly influencing the inflammation and cancer biology (Krishn, Batra, and Kaur 2015). The expression of MUC2 and TFF3 have been reported to be posttranscriptionally affected by the interaction of ncRNAs (Zhou et al. 2016; Hanisch et al. 2017). In the current study, miR-125a-5p (0.72-fold change compared with control) and miR-615-3p (0.84-fold change compared with control) possess anti-correlated expression with MUC2 and TFF3 in transcription level during *C. jejuni* infection *in vitro*. In addition, an anti-correlation between MUC2/TFF3 and miR-615-3p (3.10-fold change compared with control) in transcription level was observed in mouse colon tissue upon *C. jejuni* infection *in vivo*. These findings may indicate the regulatory importance of miR-125a-5p and miR-615-3p on mucin in the context of *C. jejuni* infection. However, more investigation is required to confirm whether there are the specific targeting relationships between miR-125a-5p or miR-615-3p and MUC2/TFF3 in the context of *C. jejuni* infection.

Furthermore, miRNAs are suggested as major regulator of glycan biosynthesis. MiRNAs play a pivotal role in controlling the levels of specific glycosyltransferases, thereby modulating glycosylation (Kasper, Koppolu, and Mahal 2014; Agrawal et al. 2014). Several glycosyltransferases in human were verified as miRNA targets, for example, ST3GAL5, ST6GALNAc5, C1GALT1, *N*-acetyl-galactosaminyltransferase (GALNT1), fucosyltransferase 8 (FUT8). The cell-specific targeting relationship of miRNAs with these glycosyltransferases influences multiple cell functions ranging from immune responses, malignance and metastasis

to cell cycle, proliferation and apoptosis, etc. (Kurcon et al. 2015; Serino et al. 2012; Gaziel-Sovran et al. 2011; Bernardi et al. 2013). However, it is still unclear how pathogen infection manipulates the regulatory network concerted by miRNA to alter the expression of glycosyltransferases. Based on the *in-silico* analysis in this study, ST3GAL1, ST3GAL2, GCNT1 and GCNT4 are potential regulated candidates of miRNA-125a-5p and miRNA-615-3p. RT-qPCR analysis verified that ST3GAL1 and GCNT4 exhibited an anti-correlated expression in transcription level with miRNA-125a-5p (0.64-fold change compared with control) in the context of *C. jejuni* infection in mice *in vivo*. Similarly, ST3GAL2 and GCNT1 showed anti-correlated transcript level with miRNA-615-3p. These findings may point in that *C. jejuni* infection affects the transcriptional expression of these glycosyltransferase genes in a miRNA-dependent manner in the mouse colon. Moreover, results of luciferase reporter assay and RNA interference (RNAi) assay performed in CMT 93 cells demonstrated that miR-615-3p specifically targets gene ST3GAL2. After RNAi, a significantly increased (4.3-fold change compared with control) cellular miR-615-3p concentration was accompanied by a significant drop in the expression of ST3GAL2 mRNA (0.74-fold change compared with control). In correspondence with the dual luciferase reporter assay, miR-615-3p mimics repressed luciferase activity by around 0.65-fold compared to nonsense transfected controls. As such, miR-615-3p may play a regulatory role during the *C. jejuni* infection in mice by targeting ST3GAL2. However, whether miRNA-615-3p specifically targets GCNT1, miRNA-125a-5p specifically targets ST3GAL1 and GCNT4, as well as how these interactions affect the mucin structure in the context of *C. jejuni* infection remain to be investigated in the future.

Overall, *C. jejuni* infection induced dysregulation of MUC2 and its co-secreted peptide TFF3 at transcriptional level. The *C. jejuni* infection also caused significant alteration in posttranscriptional modification of MUC2, which is possibly manipulated by miRNA-mediated regulatory networks. Nevertheless, there are still outstanding questions remain in regard to how the interaction of miRNA-615-3p with ST3GAL2/GCNT1 functions in response to bacterial infection and how the miRNA-regulated glycosyltransferases impact the structure and composition of mucin during *C. jejuni* infection. Moreover, further work is necessary to clarify the contribution of ST3GAL1, ST3GAL2, GCNT1 and GCNT4 to mucin biosynthesis and structural formation.

6. Conclusion

The findings of the present study allow to conclude, firstly, *C. jejuni* gene *cj1492c* annotated as a two-component sensor and gene *cj1507c* described as a regulatory protein are found to be involved in various processes related to pathogenicity of *C. jejuni* in HT-29/B6 cells. Signal sensing by Cj1492c leads to restricted attachment and therefore limiting internalisation of *C. jejuni* but enhancing intracellular survival. Genes regulated by Cj1507c have an impact on efficient adhesion and invasion and also intracellular survival of *C. jejuni*. However, mutagenesis of both genes seems not to affect the host proinflammatory response as well as alteration of cell morphology.

From the aspect of host response, *C. jejuni* infection induced dysregulation of the mucin MUC2 and its mediator TFF3 as well as mucin-associated miRNAs (miR-125a-5p and miR-615-3p) at transcriptional level. Moreover, the aberrant expression of sialyltransferases ST3GAL1, ST3GAL2 that are involved in mucin *O*-type biosynthesis were observed in the mouse colon of stably infected *C. jejuni*. These mucin-associated factors seem to interact in a coordinated manner concerted by a miRNA-dependent regulatory network during *C. jejuni* infection. Among them, ST3GAL2 has been identified as target genes of miR-615-3p *in vitro*. As such, the present study suggested that the regulatory relationship of miR-615-3p-ST3GAL2 was involved in the host cellular response to *C. jejuni* infection.

To complement these results, genetic complementation of the corresponding wild-type gene might exclude polar effects and more functional study would decipher the functional effect of Cj1492c and Cj1507c in pathogenicity of *C. jejuni*. A more complete understanding of the function of Cj1492c and Cj1507c as well as the regulatory networks will provide more insights into the internalisation, *in vivo* growth and the progression to disease. Moreover, more studies are required to elucidate the mechanism by which the alteration in mucin and posttranscriptional modification are coordinated with the regulation of miRNAs during *C. jejuni* infection. Further functional analysis could describe the miR-615-3p-ST3GAL2-interaction in response to bacterial infections in more detail. Furthermore, it could be intriguing to know the possible impact of miRNA-regulated sialyltransferases and β1, 6-*N*-acetylglucosaminyl-transferase on mucin structure during *C. jejuni* infection.

7. Summary

Identification and characterisation of *Campylobacter jejuni* novel virulence factors and mucin-associated regulatory factors relevant to the infection process

C. jejuni is an important zoonotic pathogen causing enterocolitis in humans worldwide. Multiple factors including motility, adhesion, invasion, intracellular survival and subversion of host cell responses together constitute the virulence machinery of *C. jejuni*. Among them, adhesion and invasion are thought to be the essential features for pathogenesis of *C. jejuni*. To establish an infection, *C. jejuni* have to bypass the physical and immunological barriers of the gastrointestinal tract. The mucus layer of the gastrointestinal tract epithelium serves as the first line of host defence. However, *C. jejuni* have evolved several traits to subvert or avoid this barrier and directly access the intestinal epithelium. Therefore, the interaction of *C. jejuni* and mucus is recognised as a critical process in the colonisation of mucosal surfaces. Herein, the specific binding between bacteria and host via “glycan-glycan talk” constitutes a prominent mechanism for bacteria to mediate adhesion and invasion of host cells. During infection, mucin regulation and posttranscriptional modification are mediated by multitude of factors, in which the regulatory miRNA networks play a prominent role.

To identify further virulence associated genes, 24 transposon insertion mutants were screened for the ability to enter cultured epithelial cells HT-29/B6 using the established invasion assay. Two mutants with altered capability to invade human intestinal epithelial cells have been selected and used for further phenotypical characterisation. Moreover, the well-established *in vivo* murine infection model and *in vitro* human cells infection model were used to study host mucin responses to *C. jejuni* infection as well as to identify the potential regulatory network concerted by miRNAs in this context.

The results indicated that genes regulated by Cj1507c have an impact on efficient adhesion, invasion and intracellular survival of *C. jejuni* in HT-29/B6 cells. Genes regulated by Cj1492c lead to restricted attachment and hence internalisation of *C. jejuni*. Also, signal sensing by Cj1492c impacts the intracellular survival capacities. Moreover, *C. jejuni* infection cause dysregulation of mucin MUC2 and its mediator TFF3 as well as the mucin-associated miRNAs at transcriptional level. *C. jejuni* infection also induced altered gene expression of the glycosyltransferases ST3GAL1 and ST3GAL2 involved in mucin type *O*-glycan biosynthesis. These mucin-associated factors were found to interact in a coordinated manner concerted by a miRNA-dependent regulatory network during *C. jejuni* infection. Additional RNAi and

luciferase reporter assay provide evidence that the regulatory relationship of miR-615-3p and ST3GAL2 was involved in the host cellular response to *C. jejuni* infection.

8. Zusammenfassung

Identifizierung und Charakterisierung neuartiger Virulenzfaktoren von *Campylobacter jejuni* und relevante mucin-assoziierte regulatorische Faktoren im Infektionsprozess

C. jejuni ist ein weltweit relevanter zoonotischer Erreger, der bei Menschen eine Enterokolitis verursachen kann. Durch das Zusammenwirken verschiedener Faktoren wie Motilität, Adhäsion, Invasion, intrazelluläres Überleben sowie Subversion der Wirtszellantworten, ist es *C. jejuni* möglich, seine Virulenz zu behaupten. Die Adhäsion und Invasion werden als essenzielle Faktoren der Pathogenese von *C. jejuni* betrachtet. Um eine Infektion zu verursachen, ist es notwendig, dass *C. jejuni* die physischen bzw. immunologischen Barrieren des Magen-Darm-Trakts umgeht. Die Mukusschicht des Darmepithels stellt die erste Barriere der Abwehr gegen pathogene Erreger dar. *C. jejuni* hat nun verschiedene Strategien entwickelt, um diese Barrieren zu umgehen, um sich Zugang zum Darmepithel zu verschaffen. Diese Interaktion zwischen *C. jejuni* und der Mukusschicht wird als ein entscheidender Prozess zur Besiedelung der Schleimhautoberfläche der Gastrointestinaltraktes betrachtet. Durch den sogenannten „Glycan-Glycan-Talk“ entsteht eine spezifische Bindung zwischen dem Erreger und dem Wirt. Dieser Mechanismus ist bedeutend und ermöglich dem Erreger eine Adhäsion und Invasion der Wirtszellen. Während der Infektion werden die Mucinregulation und die posttranscriptionale Modifikation durch verschiedenste Faktoren vermittelt. Hierbei spielen die regulatorischen miRNA-Netzwerke eine wesentliche Rolle.

Um weitere Virulenzgene zu identifizieren, wurden unter Verwendung eines etablierten Invasionsassays 24 Transposon-Insertionsmutanten auf die Fähigkeit untersucht, in kultivierte HT-29/B6 Epithelzellen zu invadieren. Aus dieser Untersuchung wurden zwei Mutanten mit veränderter Invasionsfähigkeit in menschliche Darmepithelzellen ausgewählt und weitergehend phänotypisch charakterisiert. Darüber hinaus wurden in dieser Studie ein gut etabliertes *in vivo* Mausinfektionsmodell und ein *in vitro* Infektionsmodell menschlicher Zellen verwendet, um die Mucin-Reaktionen des Wirtes auf eine mögliche *C. jejuni*-Infektion zu untersuchen und das potenzielle regulatorische Netzwerk zu identifizieren, das von miRNAs in diesem Zusammenhang konzertiert wurde.

Die Ergebnisse dieser Studie deuten darauf hin, dass die Gene, die durch Cj1507c reguliert wurden, die Effizienz der Adhäsion und Invasion sowie das intrazelluläre Überleben von *C. jejuni* in HT-29/B6-Zellen beeinflussen. Die durch Cj1492c regulierten Gene führen zu einer eingeschränkten Anhaftung bzw. Adhäsion und somit zu einer Internalisierung von *C. jejuni*. Zudem hat sich in dieser Studie erwiesen, dass die Signalerfassung durch Cj1492c ebenfalls

die intrazelluläre Überlebenskapazität der *C. jejuni beeinflusst*. Ferner führt die Infektion durch *C. jejuni* zu einer Dysregulation von Mucin MUC2 und der entsprechenden Mediator TFF3 sowie der Mucin-assoziierten miRNAs auf Transkriptionsebene. Die Infektion durch *C. jejuni* führt auch zu einer veränderten Genexpression der Glycosyltransferases ST3GAL1 und ST3GAL2, die an der Biosynthese von Mucin-Typ-*O*-Glycan beteiligt sind. Während der *C. jejuni*-Infektion interagieren diese Mucin-assoziierten Faktoren auf eine koordinierte Weise, die durch ein miRNA-abhängiges regulatorisches Netzwerk konzertiert wird. Zusätzlich deuteten RNAi und Luciferase-Reporter-Assays darauf hin, dass die regulatorische Beziehung zwischen miR-615-3p und ST3GAL2 in der Wirtszellantwort einer *C. jejuni*-Infektion beteiligt sind.

9. References

Abreu, M. T. 2010. 'Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function', *Nat Rev Immunol*, 10: 131-44.

Agrawal, P., T. Kurcon, K. T. Pilobello, J. F. Rakus, S. Koppolu, Z. Liu, B. S. Batista, W. S. Eng, K. L. Hsu, Y. Liang, and L. K. Mahal. 2014. 'Mapping posttranscriptional regulation of the human glycome uncovers microRNA defining the glycocode', *Proc Natl Acad Sci U S A*, 111: 4338-43.

Aguilar, C., A. Jimenez-Marin, R. P. Martins, and J. J. Garrido. 2014. 'Interaction between Campylobacter and intestinal epithelial cells leads to a different proinflammatory response in human and porcine host', *Vet Immunol Immunopathol*, 162: 14-23.

Aihara, E., K. A. Engevik, and M. H. Montrose. 2017. 'Trefoil Factor Peptides and Gastrointestinal Function', *Annu Rev Physiol*, 79: 357-80.

Al-Banna, N. A., F. Cyprian, and M. J. Albert. 2018. 'Cytokine responses in campylobacteriosis: Linking pathogenesis to immunity', *Cytokine Growth Factor Rev*, 41: 75-87.

Alemka, A., N. Corcionivoschi, and B. Bourke. 2012. 'Defense and adaptation: the complex inter-relationship between Campylobacter jejuni and mucus', *Front Cell Infect Microbiol*, 2: 15.

Allaire, J. M., S. M. Crowley, H. T. Law, S. Y. Chang, H. J. Ko, and B. A. Vallance. 2018. 'The Intestinal Epithelium: Central Coordinator of Mucosal Immunity', *Trends Immunol*, 39: 677-96.

Alter, T., S. Bereswill, G. Glunder, L. M. Haag, I. Hanel, M. M. Heimesaat, R. Lugert, S. Rautenschlein, R. M. Weber, A. E. Zautner, and U. Gross. 2011. '[Campylobacteriosis of man : livestock as reservoir for Campylobacter species]', *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz*, 54: 728-34.

An, S. Q., J. H. Allan, Y. McCarthy, M. Febrer, J. M. Dow, and R. P. Ryan. 2014. 'The PAS domain-containing histidine kinase RpfS is a second sensor for the diffusible signal factor of Xanthomonas campestris', *Mol Microbiol*, 92: 586-97.

Arike, L., J. Holmen-Larsson, and G. C. Hansson. 2017. 'Intestinal Muc2 mucin O-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases', *Glycobiology*, 27: 318-28.

Backert, S., M. Boehm, S. Wessler, and N. Tegtmeyer. 2013. 'Transmigration route of Campylobacter jejuni across polarized intestinal epithelial cells: paracellular, transcellular or both?', *Cell Commun Signal*, 11: 72.

Backert, S., and D. Hofreuter. 2013. 'Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen Campylobacter jejuni', *J Microbiol Methods*, 95: 8-23.

Barrera, G. J., G. Sanchez, and J. E. Gonzalez. 2012. 'Trefoil factor 3 isolated from human breast milk downregulates cytokines (IL8 and IL6) and promotes human beta defensin (hBD2 and hBD4) expression in intestinal epithelial cells HT-29', *Bosn J Basic Med Sci*, 12: 256-64.

Batisson, I., M. P. Guimond, F. Girard, H. An, C. Zhu, E. Oswald, J. M. Fairbrother, M. Jacques, and J. Harel. 2003. 'Characterization of the novel factor paa involved in the early steps of the adhesion mechanism of attaching and effacing Escherichia coli', *Infect Immun*, 71: 4516-25.

Bautista, M. V., Y. Chen, V. S. Ivanova, M. K. Rahimi, A. M. Watson, and M. C. Rose. 2009. 'IL-8 regulates mucin gene expression at the posttranscriptional level in lung epithelial cells', *J Immunol*, 183: 2159-66.

Bereswill, S., A. Fischer, R. Plickert, L. M. Haag, B. Otto, A. A. Kuhl, J. I. Dasti, A. E. Zautner, M. Munoz, C. Loddenkemper, U. Gross, U. B. Gobel, and M. M. Heimesaat. 2011. 'Novel murine infection models provide deep insights into the "menage a trois" of Campylobacter jejuni, microbiota and host innate immunity', *PLoS One*, 6: e20953.

Bergstrom, K. S., J. A. Guttman, M. Rumi, C. Ma, S. Bouzari, M. A. Khan, D. L. Gibson, A. W. Vogl, and B. A. Vallance. 2008. 'Modulation of intestinal goblet cell function during infection by an attaching and effacing bacterial pathogen', *Infect Immun*, 76: 796-811.

Bergstrom, K. S., and L. Xia. 2013. 'Mucin-type O-glycans and their roles in intestinal homeostasis', *Glycobiology*, 23: 1026-37.

Berkes, J., V. K. Viswanathan, S. D. Savkovic, and G. Hecht. 2003. 'Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation', *Gut*, 52: 439-51.

Bernardi, C., U. Soffientini, F. Piacente, and M. G. Tonetti. 2013. 'Effects of microRNAs on fucosyltransferase 8 (FUT8) expression in hepatocarcinoma cells', *PLoS One*, 8: e76540.

Bertoli, G., C. Cava, and I. Castiglioni. 2015. 'MicroRNAs: New Biomarkers for Diagnosis, Prognosis, Therapy Prediction and Therapeutic Tools for Breast Cancer', *Theranostics*, 5: 1122-43.

Biswas, D., U. Fernando, C. Reiman, P. Willson, A. Potter, and B. Allan. 2006. 'Effect of cytolethal distending toxin of Campylobacter jejuni on adhesion and internalization in cultured cells and in colonization of the chicken gut', *Avian Dis*, 50: 586-93.

Bolton, D. J. 2015. 'Campylobacter virulence and survival factors', *Food Microbiol*, 48: 99-108.

Bouwman, L. I., M. R. de Zoete, N. M. Bleumink-Pluym, R. A. Flavell, and J. P. van Putten. 2014. 'Inflammasome activation by Campylobacter jejuni', *J Immunol*, 193: 4548-57.

Bouwman, L. I., P. Niewold, and J. P. van Putten. 2013. 'Basolateral invasion and trafficking of Campylobacter jejuni in polarized epithelial cells', *PLoS One*, 8: e54759.

Branka, J. E., G. Vallette, A. Jarry, C. Bou-Hanna, P. Lemarre, P. N. Van, and C. L. Laboisse. 1997. 'Early functional effects of Clostridium difficile toxin A on human colonocytes', *Gastroenterology*, 112: 1887-94.

Brockhausen, I. 2014. 'Crossroads between Bacterial and Mammalian Glycosyltransferases', *Front Immunol*, 5: 492.

Bu, X., L. Li, N. Li, X. Tian, and P. Huang. 2011. 'Suppression of mucin 2 enhances the proliferation and invasion of LS174T human colorectal cancer cells', *Cell Biol Int*, 35: 1121-9.

Buelow, D. R., J. E. Christensen, J. M. Neal-McKinney, and M. E. Konkel. 2011. 'Campylobacter jejuni survival within human epithelial cells is enhanced by the secreted protein CiaI', *Mol Microbiol*, 80: 1296-312.

Burnham, P. M., and D. R. Hendrixson. 2018. 'Campylobacter jejuni: collective components promoting a successful enteric lifestyle', *Nat Rev Microbiol*, 16: 551-65.

Byrd, J. C., and R. S. Bresalier. 2004. 'Mucins and mucin binding proteins in colorectal cancer', *Cancer Metastasis Rev*, 23: 77-99.

CDC. 2021. '*Campylobacter* (Campylobacteriosis)'. https://www.cdc.gov/campylobacter/index.html.

Cole, K., M. B. Farnell, A. M. Donoghue, N. J. Stern, E. A. Svetoch, B. N. Eruslanov, L. I. Volodina, Y. N. Kovalev, V. V. Perelygin, E. V. Mitsevich, I. P. Mitsevich, V. P. Levchuk, V. D. Pokhilenko, V. N. Borzenkov, O. E. Svetoch, T. Y. Kudryavtseva, I. Reyes-Herrera, P. J. Blore, F. Solis de los Santos, and D. J. Donoghue. 2006. 'Bacteriocins reduce Campylobacter colonization and alter gut morphology in turkey poults', *Poult Sci*, 85: 1570-5.

Corfield, A. P. 2018. 'The Interaction of the Gut Microbiota with the Mucus Barrier in Health and Disease in Human', *Microorganisms*, 6.

Cortes-Marquez, A. C., S. Mendoza-Elizalde, F. Arenas-Huertero, J. Trillo-Tinoco, P. Valencia-Mayoral, A. Consuelo-Sanchez, J. Zarate-Franco, A. R. Dionicio-Avendano, J. J. Herrera-Esquivel, E. G. Recinos-Carrera, C. Colin-Valverde, S. Rivera-Gutierrez, A. Reyes-Lopez, J. C. Vigueras-Galindo, and N. Velazquez-Guadarrama. 2018. 'Differential expression of miRNA-146a and miRNA-155 in gastritis induced by Helicobacter pylori infection in paediatric patients, adults, and an animal model', *BMC Infect Dis*, 18: 463.

Cróinín, T, O. , and S. Backert. 2012. 'Host epithelial cell invasion by Campylobacter jejuni: trigger or zipper mechanism?', *Front Cell Infect Microbiol*, 2: 25.

Crushell, E., S. Harty, F. Sharif, and B. Bourke. 2004. 'Enteric campylobacter: purging its secrets?', *Pediatr Res*, 55: 3-12.

Cullen, T. W., and M. S. Trent. 2010. 'A link between the assembly of flagella and lipooligosaccharide of the Gram-negative bacterium Campylobacter jejuni', *Proc Natl Acad Sci U S A*, 107: 5160-5.

Dalziel, M., C. Whitehouse, I. McFarlane, I. Brockhausen, S. Gschmeissner, T. Schwientek, H. Clausen, J. M. Burchell, and J. Taylor-Papadimitriou. 2001. 'The relative activities of the C2GnT1 and ST3Gal-I glycosyltransferases determine O-glycan structure and expression of a tumor-associated epitope on MUC1', *J Biol Chem*, 276: 11007-15.

Dasti, J. I., A. M. Tareen, R. Lugert, A. E. Zautner, and U. Gross. 2010. 'Campylobacter jejuni: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms', *Int J Med Microbiol*, 300: 205-11.

Day, C. J., E. A. Semchenko, and V. Korolik. 2012. 'Glycoconjugates play a key role in Campylobacter jejuni Infection: interactions between host and pathogen', *Front Cell Infect Microbiol*, 2: 9.

Deplancke, B., and H. R. Gaskins. 2001. 'Microbial modulation of innate defense: goblet cells and the intestinal mucus layer', *Am J Clin Nutr*, 73: 1131S-41S.

Desai, M. S., A. M. Seekatz, N. M. Koropatkin, N. Kamada, C. A. Hickey, M. Wolter, N. A. Pudlo, S. Kitamoto, N. Terrapon, A. Muller, V. B. Young, B. Henrissat, P. Wilmes, T. S. Stappenbeck, G. Nunez, and E. C. Martens. 2016. 'A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility', *Cell*, 167: 1339-53 e21.

Dossinger, V., T. Kayademir, N. Blin, and P. Gott. 2002. 'Down-regulation of TFF expression in gastrointestinal cell lines by cytokines and nuclear factors', *Cell Physiol Biochem*, 12: 197-206.

Duan, L. L., P. Guo, Y. Zhang, and H. L. Chen. 2005. 'Regulation of metastasis-suppressive gene Nm23-H1 on glycosyl-transferases involved in the synthesis of sialyl Lewis antigens', *J Cell Biochem*, 94: 1248-57.

Dufour, V., J. Li, A. Flint, E. Rosenfeld, K. Rivoal, S. Georgeault, B. Alazzam, G. Ermel, A. Stintzi, M. Bonnaure-Mallet, and C. Baysse. 2013. 'Inactivation of the LysR regulator Cj1000 of Campylobacter jejuni affects host colonization and respiration', *Microbiology*, 159: 1165-78.

ECDC, EFSA;. 2021. 'The European Union One Health 2019 Zoonoses Report', *EFSA J*, 19: e06406.

EFSA. 2016. 'The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015', *EFSA J*, 14: 4634.

EFSA, and ECDC. 2019. 'The European Union One Health 2018 Zoonoses Report', *EFSA J*, 17: e05926.

El-Battari, A., M. Prorok, K. Angata, S. Mathieu, M. Zerfaoui, E. Ong, M. Suzuki, D. Lombardo, and M. Fukuda. 2003. 'Different glycosyltransferases are differentially processed for secretion, dimerization, and autoglycosylation', *Glycobiology*, 13: 941-53.

Ellies, L. G., M. Sperandio, G. H. Underhill, J. Yousif, M. Smith, J. J. Priatel, G. S. Kansas, K. Ley, and J. D. Marth. 2002. 'Sialyltransferase specificity in selectin ligand formation', *Blood*, 100: 3618-25.

Enss, M. L., M. Cornberg, S. Wagner, A. Gebert, M. Henrichs, R. Eisenblatter, W. Beil, R. Kownatzki, and H. J. Hedrich. 2000. 'Proinflammatory cytokines trigger MUC gene expression and mucin release in the intestinal cancer cell line LS180', *Inflamm Res*, 49: 162-9.

Eulalio, A., L. Schulte, and J. Vogel. 2012. 'The mammalian microRNA response to bacterial infections', *RNA Biol*, 9: 742-50.

Eyking, A., H. Reis, M. Frank, G. Gerken, K. W. Schmid, and E. Cario. 2016. 'MiR-205 and MiR-373 Are Associated with Aggressive Human Mucinous Colorectal Cancer', *PLoS One*, 11: e0156871.

Fauchere, J. L., A. Rosenau, M. Veron, E. N. Moyen, S. Richard, and A. Pfister. 1986. 'Association with HeLa cells of Campylobacter jejuni and Campylobacter coli isolated from human feces', *Infect Immun*, 54: 283-7.

Flanagan, R. C., J. M. Neal-McKinney, A. S. Dhillon, W. G. Miller, and M. E. Konkel. 2009. 'Examination of Campylobacter jejuni putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization', *Infect Immun*, 77: 2399-407.

Freitag, C. M., K. Strijbis, and J. P. M. van Putten. 2017. 'Host cell binding of the flagellar tip protein of Campylobacter jejuni', *Cell Microbiol*, 19.

Fu, J., B. Wei, T. Wen, M. E. Johansson, X. Liu, E. Bradford, K. A. Thomsson, S. McGee, L. Mansour, M. Tong, J. M. McDaniel, T. J. Sferra, J. R. Turner, H. Chen, G. C. Hansson, J. Braun, and L. Xia. 2011. 'Loss of intestinal core 1-derived O-glycans causes spontaneous colitis in mice', *J Clin Invest*, 121: 1657-66.

Gaziel-Sovran, A., M. F. Segura, R. Di Micco, M. K. Collins, D. Hanniford, E. Vega-Saenz de Miera, J. F. Rakus, J. F. Dankert, S. Shang, R. S. Kerbel, N. Bhardwaj, Y. Shao, F. Darvishian, J. Zavadil, A. Erlebacher, L. K. Mahal, I. Osman, and E. Hernando. 2011. 'miR-30b/30d regulation of GalNAc transferases enhances invasion and immunosuppression during metastasis', *Cancer Cell*, 20: 104-18.

Gebert, L. F. R., and I. J. MacRae. 2019. 'Regulation of microRNA function in animals', *Nat Rev Mol Cell Biol*, 20: 21-37.

Golden, N. J., and D. W. Acheson. 2002. 'Identification of motility and autoagglutination Campylobacter jejuni mutants by random transposon mutagenesis', *Infect Immun*, 70: 1761-71.

Golz, G., B. Rosner, D. Hofreuter, C. Josenhans, L. Kreienbrock, A. Lowenstein, A. Schielke, K. Stark, S. Suerbaum, L. H. Wieler, and T. Alter. 2014. 'Relevance of Campylobacter to public health--the need for a One Health approach', *Int J Med Microbiol*, 304: 817-23.

Gomes, J., A. Magalhaes, A. S. Carvalho, G. E. Hernandez, S. L. Papp, S. R. Head, V. Michel, L. David, F. Gartner, E. Touati, and C. A. Reis. 2012. 'Glycophenotypic alterations induced by Pteridium aquilinum in mice gastric mucosa: synergistic effect with Helicobacter pylori infection', *PLoS One*, 7: e38353.

Goon, S., C. P. Ewing, M. Lorenzo, D. Pattarini, G. Majam, and P. Guerry. 2006. 'A sigma28-regulated nonflagella gene contributes to virulence of Campylobacter jejuni 81-176', *Infect Immun*, 74: 769-72.

Guerry, P. 2007. 'Campylobacter flagella: not just for motility', *Trends Microbiol*, 15: 456-61.

Gupta, R., F. Leon, S. Rauth, S. K. Batra, and M. P. Ponnusamy. 2020. 'A Systematic Review on the Implications of O-linked Glycan Branching and Truncating Enzymes on Cancer Progression and Metastasis', *Cells*, 9.

Haag, L. M., A. Fischer, B. Otto, R. Plickert, A. A. Kuhl, U. B. Gobel, S. Bereswill, and M. M. Heimesaat. 2012. 'Campylobacter jejuni induces acute enterocolitis in gnotobiotic IL-10-/- mice via Toll-like-receptor-2 and -4 signaling', *PLoS One*, 7: e40761.

Haiko, J., and B. Westerlund-Wikstrom. 2013. 'The role of the bacterial flagellum in adhesion and virulence', *Biology (Basel)*, 2: 1242-67.

Hameed, A., A. Woodacre, L. R. Machado, and G. L. Marsden. 2020. 'An Updated Classification System and Review of the Lipooligosaccharide Biosynthesis Gene Locus in Campylobacter jejuni', *Front Microbiol*, 11: 677.

Hanisch, C., J. Sharbati, B. Kutz-Lohroff, O. Huber, R. Einspanier, and S. Sharbati. 2017. 'TFF3-dependent resistance of human colorectal adenocarcinoma cells HT-29/B6 to apoptosis is mediated by miR-491-5p regulation of lncRNA PRINS', *Cell Death Discov*, 3: 16106.

Hansson, G. C. 2012. 'Role of mucus layers in gut infection and inflammation', *Curr Opin Microbiol*, 15: 57-62.

Hansson, G. C., and M. E. Johansson. 2010. 'The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria', *Gut Microbes*, 1: 51-54.

Heimesaat, M. M., S. Bereswill, A. Fischer, D. Fuchs, D. Struck, J. Niebergall, H. K. Jahn, I. R. Dunay, A. Moter, D. M. Gescher, R. R. Schumann, U. B. Gobel, and O. Liesenfeld. 2006. 'Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii', *J Immunol*, 177: 8785-95.

Heimesaat, M. M., U. Grundmann, M. E. Alutis, A. Fischer, and S. Bereswill. 2017. 'Absence of Nucleotide-Oligomerization-Domain-2 Is Associated with Less Distinct Disease in Campylobacter jejuni Infected Secondary Abiotic IL-10 Deficient Mice', *Front Cell Infect Microbiol*, 7: 322.

Hendrixson, D. R., and V. J. DiRita. 2004. 'Identification of Campylobacter jejuni genes involved in commensal colonization of the chick gastrointestinal tract', *Mol Microbiol*, 52: 471-84.

Heroven, A. K., and P. Dersch. 2006. 'RovM, a novel LysR-type regulator of the virulence activator gene rovA, controls cell invasion, virulence and motility of Yersinia pseudotuberculosis', *Mol Microbiol*, 62: 1469-83.

Hoeke, L., J. Sharbati, K. Pawar, A. Keller, R. Einspanier, and S. Sharbati. 2013. 'Intestinal Salmonella typhimurium infection leads to miR-29a induced caveolin 2 regulation', *PLoS One*, 8: e67300.

Holmen Larsson, J. M., K. A. Thomsson, A. M. Rodriguez-Pineiro, H. Karlsson, and G. C. Hansson. 2013. 'Studies of mucus in mouse stomach, small intestine, and colon. III. Gastrointestinal Muc5ac and Muc2 mucin O-glycan patterns reveal a regiospecific distribution', *Am J Physiol Gastrointest Liver Physiol*, 305: G357-63.

Hu, L., and D. J. Kopecko. 1999. 'Campylobacter jejuni 81-176 associates with microtubules and dynein during invasion of human intestinal cells', *Infect Immun*, 67: 4171-82.

Iwashita, J., Y. Sato, H. Sugaya, N. Takahashi, H. Sasaki, and T. Abe. 2003. 'mRNA of MUC2 is stimulated by IL-4, IL-13 or TNF-alpha through a mitogen-activated protein kinase pathway in human colon cancer cells', *Immunol Cell Biol*, 81: 275-82.

Janssen, R., K. A. Krogfelt, S. A. Cawthraw, W. van Pelt, J. A. Wagenaar, and R. J. Owen. 2008. 'Host-pathogen interactions in Campylobacter infections: the host perspective', *Clin Microbiol Rev*, 21: 505-18.

Javed, M. A., A. J. Grant, M. C. Bagnall, D. J. Maskell, D. G. Newell, and G. Manning. 2010. 'Transposon mutagenesis in a hyper-invasive clinical isolate of Campylobacter jejuni reveals a number of genes with potential roles in invasion', *Microbiology*, 156: 1134-43.

Jensen, P. H., D. Kolarich, and N. H. Packer. 2010. 'Mucin-type O-glycosylation--putting the pieces together', *FEBS J*, 277: 81-94.

Johansson, M. E., D. Ambort, T. Pelaseyed, A. Schutte, J. K. Gustafsson, A. Ermund, D. B. Subramani, J. M. Holmen-Larsson, K. A. Thomsson, J. H. Bergstrom, S. van der Post, A. M. Rodriguez-Pineiro, H. Sjovall, M. Backstrom, and G. C. Hansson. 2011.

'Composition and functional role of the mucus layers in the intestine', *Cell Mol Life Sci*, 68: 3635-41.

Johansson, M. E., M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G. C. Hansson. 2008. 'The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria', *Proc Natl Acad Sci U S A*, 105: 15064-9.

Johansson, M. E., H. Sjovall, and G. C. Hansson. 2013. 'The gastrointestinal mucus system in health and disease', *Nat Rev Gastroenterol Hepatol*, 10: 352-61.

John, Daniel A., Lisa K. Williams, Venkateswarlu Kanamarlapudi, Thomas J. Humphrey, and Thomas S. Wilkinson. 2017. 'The Bacterial Species Campylobacter jejuni Induce Diverse Innate Immune Responses in Human and Avian Intestinal Epithelial Cells', 8.

Karlyshev, A. V., J. M. Ketley, and B. W. Wren. 2005. 'The Campylobacter jejuni glycome', *FEMS Microbiol Rev*, 29: 377-90.

Kasper, B. T., S. Koppolu, and L. K. Mahal. 2014. 'Insights into miRNA regulation of the human glycome', *Biochem Biophys Res Commun*, 445: 774-9.

Kim, Y. S., and S. B. Ho. 2010. 'Intestinal goblet cells and mucins in health and disease: recent insights and progress', *Curr Gastroenterol Rep*, 12: 319-30.

Kindon, H., C. Pothoulakis, L. Thim, K. Lynch-Devaney, and D. K. Podolsky. 1995. 'Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein', *Gastroenterology*, 109: 516-23.

Konkel, M. E., B. J. Kim, V. Rivera-Amill, and S. G. Garvis. 1999. 'Bacterial secreted proteins are required for the internaliztion of Campylobacter jejuni into cultured mammalian cells', *Mol Microbiol*, 32: 691-701.

Konkel, M. E., D. J. Mead, S. F. Hayes, and W. Cieplak, Jr. 1992. 'Translocation of Campylobacter jejuni across human polarized epithelial cell monolayer cultures', *J Infect Dis*, 166: 308-15.

Koolman, L., P. Whyte, C. Burgess, and D. Bolton. 2016. 'Virulence gene expression, adhesion and invasion of Campylobacter jejuni exposed to oxidative stress (H2O2)', *Int J Food Microbiol*, 220: 33-8.

Kovacs, J. K., A. Cox, B. Schweitzer, G. Maroti, T. Kovacs, H. Fenyvesi, L. Emody, and G. Schneider. 2020. 'Virulence Traits of Inpatient Campylobacter jejuni Isolates, and a Transcriptomic Approach to Identify Potential Genes Maintaining Intracellular Survival', *Microorganisms*, 8.

Kreisman, L. S., and B. A. Cobb. 2012. 'Infection, inflammation and host carbohydrates: a Glyco-Evasion Hypothesis', *Glycobiology*, 22: 1019-30.

Kreling, V., F. H. Falcone, C. Kehrenberg, and A. Hensel. 2020. 'Campylobacter sp.: Pathogenicity factors and prevention methods-new molecular targets for innovative antivirulence drugs?', *Appl Microbiol Biotechnol*, 104: 10409-36.

Kreusel, K. M., M. Fromm, J. D. Schulzke, and U. Hegel. 1991. 'Cl- secretion in epithelial monolayers of mucus-forming human colon cells (HT-29/B6)', *Am J Physiol*, 261: C574-82.

Krishn, S. R., S. K. Batra, and S. Kaur. 2015. 'Advances in miRNA-Mediated Mucin Regulation', *Curr Pharmacol Rep*, 1: 355-64.

Kudelka, M. R., S. R. Stowell, R. D. Cummings, and A. S. Neish. 2020. 'Intestinal epithelial glycosylation in homeostasis and gut microbiota interactions in IBD', *Nat Rev Gastroenterol Hepatol*, 17: 597-617.

Kurcon, T., Z. Liu, A. V. Paradkar, C. A. Vaiana, S. Koppolu, P. Agrawal, and L. K. Mahal. 2015. 'miRNA proxy approach reveals hidden functions of glycosylation', *Proc Natl Acad Sci U S A*, 112: 7327-32.

Larsson, J. M., H. Karlsson, J. G. Crespo, M. E. Johansson, L. Eklund, H. Sjovall, and G. C. Hansson. 2011. 'Altered O-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation', *Inflamm Bowel Dis*, 17: 2299-307.

Lavelle, E. C., C. Murphy, L. A. O'Neill, and E. M. Creagh. 2010. 'The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis', *Mucosal Immunol*, 3: 17-28.

Le, T., C. O'Brien, U. Gupta, G. Sousa, P. Daoutidis, and W. S. Hu. 2019. 'An integrated platform for mucin-type O-glycosylation network generation and visualization', *Biotechnol Bioeng*, 116: 1341-54.

Lee, H. J., L. L. Lairson, J. R. Rich, E. Lameignere, W. W. Wakarchuk, S. G. Withers, and N. C. Strynadka. 2011. 'Structural and kinetic analysis of substrate binding to the sialyltransferase Cst-II from Campylobacter jejuni', *J Biol Chem*, 286: 35922-32.

Lehnen, D., C. Blumer, T. Polen, B. Wackwitz, V. F. Wendisch, and G. Unden. 2002. 'LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in Escherichia coli', *Mol Microbiol*, 45: 521-32.

Linden, S. K., P. Sutton, N. G. Karlsson, V. Korolik, and M. A. McGuckin. 2008. 'Mucins in the mucosal barrier to infection', *Mucosal Immunol*, 1: 183-97.

Liu, L., W. Saitz-Rojas, R. Smith, L. Gonyar, J. G. In, O. Kovbasnjuk, N. C. Zachos, M. Donowitz, J. P. Nataro, and F. Ruiz-Perez. 2020. 'Mucus layer modeling of human colonoids during infection with enteroaggragative E. coli', *Sci Rep*, 10: 10533.

Liu, Z., X. Chen, Q. Wu, J. Song, L. Wang, and G. Li. 2016. 'miR-125b inhibits goblet cell differentiation in allergic airway inflammation by targeting SPDEF', *Eur J Pharmacol*, 782: 14-20.

Lopez, P. H., S. Aja, K. Aoki, M. M. Seldin, X. Lei, G. V. Ronnett, G. W. Wong, and R. L. Schnaar. 2017. 'Mice lacking sialyltransferase ST3Gal-II develop late-onset obesity and insulin resistance', *Glycobiology*, 27: 129-39.

Luethy, P. M., S. Huynh, C. T. Parker, and D. R. Hendrixson. 2015. 'Analysis of the activity and regulon of the two-component regulatory system composed by Cjj81176_1484 and Cjj81176_1483 of Campylobacter jejuni', *J Bacteriol*, 197: 1592-605.

Ma, X. H., H. J. Ren, R. Y. Peng, Y. Li, and L. Ming. 2020. 'Comparative expression profiles of host circulating miRNAs in response to Trichinella spiralis infection', *Vet Res*, 51: 39.

Maddocks, S. E., and P. C. Oyston. 2008. 'Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins', *Microbiology*, 154: 3609-23.

Mansfield, L. S., J. S. Patterson, B. R. Fierro, A. J. Murphy, V. A. Rathinam, J. J. Kopper, N. I. Barbu, T. J. Onifade, and J. A. Bell. 2008. 'Genetic background of IL-10(-/-) mice alters host-pathogen interactions with Campylobacter jejuni and influences disease phenotype', *Microb Pathog*, 45: 241-57.

Masanta, W. O., M. M. Heimesaat, S. Bereswill, A. M. Tareen, R. Lugert, U. Gross, and A. E. Zautner. 2013. 'Modification of intestinal microbiota and its consequences for innate immune response in the pathogenesis of campylobacteriosis', *Clin Dev Immunol*, 2013: 526860.

Morita, H., M. G. Kettlewell, D. P. Jewell, and P. W. Kent. 1993. 'Glycosylation and sulphation of colonic mucus glycoproteins in patients with ulcerative colitis and in healthy subjects', *Gut*, 34: 926-32.

Mousavi, S., U. Escher, E. Thunhorst, S. Kittler, C. Kehrenberg, S. Bereswill, and M. M. Heimesaat. 2020. 'Vitamin C alleviates acute enterocolitis in Campylobacter jejuni infected mice', *Sci Rep*, 10: 2921.

Nachamkin, I., X. H. Yang, and N. J. Stern. 1993. 'Role of Campylobacter jejuni flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants', *Appl Environ Microbiol*, 59: 1269-73.

Naughton, J. A., K. Marino, B. Dolan, C. Reid, R. Gough, M. E. Gallagher, M. Kilcoyne, J. Q. Gerlach, L. Joshi, P. Rudd, S. Carrington, B. Bourke, and M. Clyne. 2013. 'Divergent mechanisms of interaction of Helicobacter pylori and Campylobacter jejuni with mucus and mucins', *Infect Immun*, 81: 2838-50.

Naughton, J., G. Duggan, B. Bourke, and M. Clyne. 2014. 'Interaction of microbes with mucus and mucins: recent developments', *Gut Microbes*, 5: 48-52.

Navarro-Garcia, F., J. Gutierrez-Jimenez, C. Garcia-Tovar, L. A. Castro, H. Salazar-Gonzalez, and V. Cordova. 2010. 'Pic, an autotransporter protein secreted by different pathogens in the Enterobacteriaceae family, is a potent mucus secretagogue', *Infect Immun*, 78: 4101-9.

Novik, V., D. Hofreuter, and J. E. Galan. 2010. 'Identification of Campylobacter jejuni genes involved in its interaction with epithelial cells', *Infect Immun*, 78: 3540-53.

Novik, Veronica, Dirk Hofreuter, and Jorge E. Galán. 2010. 'Identification of Campylobacter jejuni Genes Involved in Its Interaction with Epithelial Cells', 78: 3540-53.

Oakland, M., B. Jeon, O. Sahin, Z. Shen, and Q. Zhang. 2011. 'Functional characterization of a lipoprotein-encoding operon in Campylobacter jejuni', *PLoS One*, 6: e20084.

On, S. L. 2001. 'Taxonomy of Campylobacter, Arcobacter, Helicobacter and related bacteria: current status, future prospects and immediate concerns', *Symp Ser Soc Appl Microbiol*: 1S-15S.

On, S.L. 2013. 'Isolation, identification and subtyping of Campylobacter: where to from here?', *J Microbiol Methods*, 95: 3-7.

Parkhill, J., B. W. Wren, K. Mungall, J. M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R. M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Moule, M. J. Pallen, C. W. Penn, M. A. Quail, M. A. Rajandream, K. M. Rutherford, A. H. van Vliet, S.

Whitehead, and B. G. Barrell. 2000. 'The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences', *Nature*, 403: 665-8.

Perera, V. N., I. Nachamkin, H. Ung, J. H. Patterson, M. J. McConville, P. J. Coloe, and B. N. Fry. 2007. 'Molecular mimicry in Campylobacter jejuni: role of the lipo-oligosaccharide core oligosaccharide in inducing anti-ganglioside antibodies', *FEMS Immunol Med Microbiol*, 50: 27-36.

Pizarro-Cerda, J., and P. Cossart. 2006. 'Bacterial adhesion and entry into host cells', *Cell*, 124: 715-27.

Poole, J., C. J. Day, M. von Itzstein, J. C. Paton, and M. P. Jennings. 2018. 'Glycointeractions in bacterial pathogenesis', *Nat Rev Microbiol*, 16: 440-52.

Prati, E. G., M. Matasci, T. B. Suter, A. Dinter, A. R. Sburlati, and J. E. Bailey. 2000. 'Engineering of coordinated up- and down-regulation of two glycosyltransferases of the O-glycosylation pathway in Chinese hamster ovary (CHO) cells', *Biotechnol Bioeng*, 68: 239-44.

Prescott, J. F., I. K. Barker, K. I. Manninen, and O. P. Miniats. 1981. 'Campylobacter jejuni colitis in gnotobiotic dogs', *Can J Comp Med*, 45: 377-83.

Proenca, M. A., J. M. Biselli, M. Succi, F. E. Severino, G. N. Berardinelli, A. Caetano, R. M. Reis, D. J. Hughes, and A. E. Silva. 2018. 'Relationship between Fusobacterium nucleatum, inflammatory mediators and microRNAs in colorectal carcinogenesis', *World J Gastroenterol*, 24: 5351-65.

Pryjma, M., D. Apel, S. Huynh, C. T. Parker, and E. C. Gaynor. 2012. 'FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of Campylobacter jejuni following host cell infection', *J Bacteriol*, 194: 3803-13.

Rivera-Amill, V., B. J. Kim, J. Seshu, and M. E. Konkel. 2001. 'Secretion of the virulence-associated Campylobacter invasion antigens from Campylobacter jejuni requires a stimulatory signal', *J Infect Dis*, 183: 1607-16.

RKI. 2019. "Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten für 2018." In. Berlin.

RKI. 2020. "Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten für 2019." In. Berlin.

Rogier, E. W., A. L. Frantz, M. E. Bruno, and C. S. Kaetzel. 2014. 'Secretory IgA is Concentrated in the Outer Layer of Colonic Mucus along with Gut Bacteria', *Pathogens*, 3: 390-403.

Rotkrua, P., Y. Akiyama, Y. Hashimoto, T. Otsubo, and Y. Yuasa. 2011. 'MiR-9 downregulates CDX2 expression in gastric cancer cells', *Int J Cancer*, 129: 2611-20.

Rubinchik, S., A. Seddon, and A. V. Karlyshev. 2012. 'Molecular mechanisms and biological role of Campylobacter jejuni attachment to host cells', *Eur J Microbiol Immunol (Bp)*, 2: 32-40.

Same, R. G., and P. D. Tamma. 2018. 'Campylobacter Infections in Children', *Pediatr Rev*, 39: 533-41.

Sarkar, J., D. P. Miller, L. D. Oliver, Jr., and R. T. Marconi. 2018. 'The Treponema denticola PAS Domain-Containing Histidine Kinase Hpk2 Is a Heme Binding Sensor of Oxygen Levels', *J Bacteriol*, 200.

Schell, M. A. 1993. 'Molecular biology of the LysR family of transcriptional regulators', *Annu Rev Microbiol*, 47: 597-626.

Schmidt, A. M., U. Escher, S. Mousavi, M. Boehm, S. Backert, S. Bereswill, and M. M. Heimesaat. 2019. 'Protease Activity of Campylobacter jejuni HtrA Modulates Distinct Intestinal and Systemic Immune Responses in Infected Secondary Abiotic IL-10 Deficient Mice', *Front Cell Infect Microbiol*, 9: 79.

Serino, G., F. Sallustio, S. N. Cox, F. Pesce, and F. P. Schena. 2012. 'Abnormal miR-148b expression promotes aberrant glycosylation of IgA1 in IgA nephropathy', *J Am Soc Nephrol*, 23: 814-24.

Sharbati, J., A. Lewin, B. Kutz-Lohroff, E. Kamal, R. Einspanier, and S. Sharbati. 2011. 'Integrated microRNA-mRNA-analysis of human monocyte derived macrophages upon Mycobacterium avium subsp. hominissuis infection', *PLoS One*, 6: e20258.

Sicard, J. F., G. Le Bihan, P. Vogeleer, M. Jacques, and J. Harel. 2017. 'Interactions of Intestinal Bacteria with Components of the Intestinal Mucus', *Front Cell Infect Microbiol*, 7: 387.

Silva, J., D. Leite, M. Fernandes, C. Mena, P. A. Gibbs, and P. Teixeira. 2011. 'Campylobacter spp. as a Foodborne Pathogen: A Review', *Front Microbiol*, 2: 200.

Singh, N., E. A. Shirdel, L. Waldron, R. H. Zhang, I. Jurisica, and E. M. Comelli. 2012. 'The murine caecal microRNA signature depends on the presence of the endogenous microbiota', *Int J Biol Sci*, 8: 171-86.

Sirard, J. C., M. Bayardo, and A. Didierlaurent. 2006. 'Pathogen-specific TLR signaling in mucosa: mutual contribution of microbial TLR agonists and virulence factors', *Eur J Immunol*, 36: 260-3.

Skoog, E. C., A. Sjoling, N. Navabi, J. Holgersson, S. B. Lundin, and S. K. Linden. 2012. 'Human gastric mucins differently regulate Helicobacter pylori proliferation, gene expression and interactions with host cells', *PLoS One*, 7: e36378.

Specian, R. D., and M. G. Oliver. 1991. 'Functional biology of intestinal goblet cells', *Am J Physiol*, 260: C183-93.

Sperandio, B., N. Fischer, M. Joncquel Chevalier-Curt, Y. Rossez, P. Roux, C. Robbe Masselot, and P. J. Sansonetti. 2013. 'Virulent Shigella flexneri affects secretion, expression, and glycosylation of gel-forming mucins in mucus-producing cells', *Infect Immun*, 81: 3632-43.

Staedel, C., and F. Darfeuille. 2013. 'MicroRNAs and bacterial infection', *Cell Microbiol*, 15: 1496-507.

Stone, E. L., S. H. Lee, M. N. Ismail, and M. Fukuda. 2010. 'Characterization of mice with targeted deletion of the gene encoding core 2 beta1,6-N-acetylglucosaminyltransferase-2', *Methods Enzymol*, 479: 155-72.

Struwe, W. B., R. Gough, M. E. Gallagher, D. T. Kenny, S. D. Carrington, N. G. Karlsson, and P. M. Rudd. 2015. 'Identification of O-glycan Structures from Chicken Intestinal Mucins

Provides Insight into Campylobactor jejuni Pathogenicity', *Mol Cell Proteomics*, 14: 1464-77.

Sun, Y. M., K. Y. Lin, and Y. Q. Chen. 2013. 'Diverse functions of miR-125 family in different cell contexts', *J Hematol Oncol*, 6: 6.

Tang, Y., C. B. Forsyth, and A. Keshavarzian. 2011. 'New molecular insights into inflammatory bowel disease-induced diarrhea', *Expert Rev Gastroenterol Hepatol*, 5: 615-25.

Tareen, A. M., C. G. Luder, A. E. Zautner, U. Grobeta, M. M. Heimesaat, S. Bereswill, and R. Lugert. 2013. 'The Campylobacter jejuni Cj0268c protein is required for adhesion and invasion in vitro', *PLoS One*, 8: e81069.

Tran, D. T., and K. G. Ten Hagen. 2013. 'Mucin-type O-glycosylation during development', *J Biol Chem*, 288: 6921-9.

Tu, Q. V., M. A. McGuckin, and G. L. Mendz. 2008. 'Campylobacter jejuni response to human mucin MUC2: modulation of colonization and pathogenicity determinants', *J Med Microbiol*, 57: 795-802.

Van den Steen, P., P. M. Rudd, R. A. Dwek, and G. Opdenakker. 1998. 'Concepts and principles of O-linked glycosylation', *Crit Rev Biochem Mol Biol*, 33: 151-208.

Van Klinken, B. J., J. W. Van der Wal, A. W. Einerhand, H. A. Buller, and J. Dekker. 1999. 'Sulphation and secretion of the predominant secretory human colonic mucin MUC2 in ulcerative colitis', *Gut*, 44: 387-93.

van Vliet, A. H., and J. M. Ketley. 2001. 'Pathogenesis of enteric Campylobacter infection', *Symp Ser Soc Appl Microbiol*: 45S-56S.

Watson, R. O., and J. E. Galan. 2008. 'Campylobacter jejuni survives within epithelial cells by avoiding delivery to lysosomes', *PLoS Pathog*, 4: e14.

Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett. 1998. 'Campylobacter jejuni cytolethal distending toxin causes a G2-phase cell cycle block', *Infect Immun*, 66: 1934-40.

WHO. 2019. '23 million people falling ill from unsafe food each year in Europe is just the tip of the iceberg'. https://www.who.int/news/item/05-06-2019-23-million-people-falling-ill-from-unsafe-food-each-year-in-europe-is-just-the-tip-of-the-iceberg.

WHO. 2020. 'Campylobacter'. https://www.who.int/en/news-room/fact-sheets/detail/campylobacter.

Xue, Y., H. Zhang, H. Wang, J. Hu, M. Du, and M. J. Zhu. 2014. 'Host inflammatory response inhibits Escherichia coli O157:H7 adhesion to gut epithelium through augmentation of mucin expression', *Infect Immun*, 82: 1921-30.

Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry. 1994. 'Isolation of motile and non-motile insertional mutants of Campylobacter jejuni: the role of motility in adherence and invasion of eukaryotic cells', *Mol Microbiol*, 14: 883-93.

Yao, R., D. H. Burr, and P. Guerry. 1997. 'CheY-mediated modulation of Campylobacter jejuni virulence', *Mol Microbiol*, 23: 1021-31.

Young, K. T., L. M. Davis, and V. J. Dirita. 2007. 'Campylobacter jejuni: molecular biology and pathogenesis', *Nat Rev Microbiol*, 5: 665-79.

Zenebe, T., N. Zegeye, and T. Eguale. 2020. 'Prevalence of Campylobacter species in human, animal and food of animal origin and their antimicrobial susceptibility in Ethiopia: a systematic review and meta-analysis', *Ann Clin Microbiol Antimicrob*, 19: 61.

Zhang, P., W. Wu, Q. Chen, and M. Chen. 2019. 'Non-Coding RNAs and their Integrated Networks', *J Integr Bioinform*, 16.

Zheng, J., J. Meng, S. Zhao, R. Singh, and W. Song. 2008. 'Campylobacter-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires Campylobacter-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB', *Infect Immun*, 76: 4498-508.

Zhou, X., H. Chen, L. Zhu, B. Hao, W. Zhang, J. Hua, H. Gu, W. Jin, and G. Zhang. 2016. 'Helicobacter pylori infection related long noncoding RNA (lncRNA) AF147447 inhibits gastric cancer proliferation and invasion by targeting MUC2 and up-regulating miR-34c', *Oncotarget*, 7: 82770-82.

10. Publications and scientific activity

10.1. Publications

- **Xi, D.**, Hofmann, L., Alter, T., Einspanier, R., Bereswill, S., Heimesaat, MM., Gölz, G., Sharbati, S. (2021) The glycosyltransferase ST3GAL2 is regulated by miR-615-3p in the intestinal tract of *Campylobacter jejuni* infected mice. *Gut Pathog*. 2021 Jun 28; 13(1):42.
  DOI: https://doi.org/10.1186/s13099-021-00437-1

- **Xi, D**., Alter, T., Einspanier, R., Sharbati, S., Gölz, G. (2020) *Campylobacter jejuni* genes Cj1492c and Cj1507c are involved in host cell adhesion and invasion. *Gut Pathog*. 2020 Feb 11; 12:8.
  DOI: https://doi.org/10.1186/s13099-020-00347-8

- Baldus M., Tsushima S., **Xi D.**, Majetschak S., Methner F.-J. (2018) Response Surface and Kinetic Modeling of Dimethyl Sulfide Oxidation – On the Origin of Dimethyl Sulfoxide in Malt, *Journal of the American Society of Brewing Chemists*, **76**:1, 29-37
  DOI: https://doi.org/10.1080/03610470.2017.1403816

10.2. Scientific activities

- Talk given at DipDok-Meeting at Robert Koch-Institute, Berlin, Germany. November 1st, 2019
  Title: *Campylobacter jejuni* genes Cj1492c and Cj1507c are involved in Host Cell Adhesion and Invasion
- Poster presented at the International Symposium on Zoonoses Research, Berlin, Germany. October 16th-18th, 2019
  Title: *Campylobacter jejuni* genes Cj1492c and Cj1507c are involved in Host Cell Adhesion and Invasion
- Poster presented at the 20th *Campylobacter*, *Helicobacter* and Related Organisms Conference, Belfast, UK. September 8th-11th, 2019
  Title: *Campylobacter jejuni* genes Cj1492c and Cj1507c are involved in Host Cell Adhesion and Invasion
- Talk given at Junior Scientist Zoonoses Meeting at Robert Koch-Institute, Berlin, Germany. June 20th-22nd, 2019

Title: *Campylobacter jejuni* genes Cj1492c and Cj1507c are involved in Host Cell Adhesion and Invasion

- Poster presented at the International Symposium on Zoonoses Research, Berlin, Germany. October 17th-19th, 2018
  Title: Host response of *Campylobacter jejuni* mutants with altered invasion capability
- Talk given at 11th Doktorandensymposium & DRS Präsentationsseminar “Biomedical Sciences”. Berlin, Germany. September 21st, 2018
  Title: Host response of *Campylobacter jejuni* mutants with altered invasion capability
- Research Internship of 6th French German Summer School, Veterinärmedizinische Universität Wien, Vienna, Austria. July 2nd-14th, 2018
- Academic Internship of the Spring School at Dahlem Research School Biomedical Science, Berlin, Germany. March 6th-17th, 2017

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12. Selbstständigkeitserklärung

Hiermit bestätige ich, **De Xi,** dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den **23.09.2021** **De Xi**

13. Interessenskonflikte

Im Rahmen dieser Arbeit bestehen keine Interessenskonflikte durch Zuwendungen Dritter.

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