# Innate Immune Defence to *Campylobacter jejuni*

**By**

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# ABSTRACT

*Campylobacter jejuni* is the most prevalent cause of bacterial diarrhoea worldwide and is frequently associated with severe post-infectious complications such as the Guillain-Barré syndrome. Despite the serious health burden caused by the bacterium disease pathogenesis remains ill defined. Human β-defensins (hBDs), a family of epithelial antimicrobial peptides, are a major component of host innate defence at mucosal surfaces. In the present study we investigated the effect of *C. jejuni* on intestinal epithelial innate responses. Up-regulation of IL-8, hBD-2 and hBD-3 gene and peptide expression was observed in Caco-2 and HT-29 cell-lines in response to *C. jejuni* strains 11168H and 81-176. Furthermore, recombinant hBDs were found to exhibit potent bactericidal activity against *C. jejuni* suggesting a major role for these peptides in disease pathogenesis.

Secondly, we aimed to identify host receptor(s) involved in sensing of *C. jejuni* and initiating innate defence. Given the invasive nature of infection, we investigated the potential role of cytoplasmic nucleotide-binding oligomerisation domain (NOD) proteins. Using small interfering (si) RNA targeting NOD1 and transfection of NOD2 overexpression plasmids, we identified NOD1 as a major pattern recognition receptor involved in mediating innate host defence to *C. jejuni* while NOD2 was found to play a minor role. Additionally, reduced NOD1 expression resulted in an increased number of intracellular *C. jejuni* thus highlighting a critical role for NOD1 mediated antimicrobial defence in limiting infection.

In the final part of the study an *ex-vivo* model of *C. jejuni* infection using human intestinal biopsies was developed. Additionally, a vertical diffusion chamber system was utilised to improve culture conditions in *C. jejuni* infection models.

In conclusion, this study highlights the important role of intestinal innate host defence to *C. jejuni*. The development of new and improved models of infections has the potential to provide previously unavailable opportunities to study *C. jejuni* disease pathogenesis.

# PUBLICATIONS AND ABSTRACTS

## Publications

1. Zilbauer. M., Dorrell N., Wren B.W., Bajaj-Elliott M.
   ***Campylobacter jejuni*-mediated disease pathogenesis: an update.**
   *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2007 (in press).

2. Zilbauer, M., Dorrell N., Elmi A., Lindley K.J., Schüller, S., Jones, H.E., Klein N.J., Núñez G., Wren B. W., Bajaj-Elliott M.
   **A major role for Intestinal Epithelial Nucleotide Oligomerisation Domain 1 (NOD1) in eliciting host bactericidal immune response to *Campylobacter jejuni.*** *Cellular Microbiology,* 2007 (in press).

3. Zilbauer, M., Dorrell, N., Boughan, P.K., Harris, A., Wren, B.W., Klein, N.J., Bajaj-Elliott, M.
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## Abstracts

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   **Der intrazelluläre Rezeptor NOD1 bei der antibakteriellen Wirtsabwehr gegen *Campylobactor jejuni.***
   22th Annual meeting of the German speaking Society for Paediatric Gastroenterology and Nutrition, May 2007, Bochum, Germany.

2. Zilbauer, M., Dorrell, N., Núñez, G., Klein, N.J., Wren, B.W., Bajaj-Elliott, M.
   **The critical role of NOD1 as a major pattern recognition receptor for detection of *Campylobacter jejuni* by intestinal epithelia.**
   Young investigator research forum of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), April 2007, Schliersee, Germany.

3. Zilbauer, M., Dorrell, N., Klein, N.J., Wren, B.W., Bajaj-Elliott, M.
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1. Zilbauer, M., N. Dorrell, Elmi, A., Lindley, K.J., Núñez, G., Klein, N.J., Wren, B.W., Bajaj-Elliott, M.
**A major role for the intestinal epithelial Nucleotide Oligomerization Domain 1 (NOD1) in mediating host bactericidal activity against *Campylobacter jejuni.*** *Journal of Paediatric Gastroenterology & Nutrition.* 2007 May; 44(6):e116.
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# ABBREVIATIONS

| | |
|---|---|
| **AMPs** | **Antimicrobial peptides** |
| **CD** | **Crohn's Disease** |
| **CDT** | **Cytolethal distending toxin** |
| ***C. jejuni*** | ***Campylobacter jejuni*** |
| **CPS** | **Capsular polysaccharide** |
| **DC** | **Dendritic cell** |
| **DNA** | **Deoxyribonucleic Acid** |
| **dNTPs** | **Deoxynucleotide triphosphates** |
| **FCS** | **Foetal calf serum** |
| **FITC** | **Fluorecein isothiocyanate** |
| **GAPDH** | **Glyceraldehyde-3 phosphate dehydrogenase** |
| **GBS** | **Guillain-Barré Syndrome** |
| **GI** | **Gastrointestinal** |
| ***H. pylori*** | ***Helicobacter pylori*** |
| **hBD** | **Human beta defensin** |
| **HD** | **Human defensin** |
| **HIV** | **Human immunodeficiency virus** |
| **HNP** | **Human neutrophil defensin** |
| **IBD** | **Inflammatory bowel disease** |
| **IEC** | **Intestinal epithelium cell** |
| **IFNγ** | **Interferon gamma** |
| **L** | **Litre** |
| **IL** | **Interleukin** |
| **IL-1RA** | **Interleukin-1 Receptor Antagonist** |

| | |
|---|---|
| **IRAK** | **Interleukin-1 Receptor -associated kinase** |
| **IVOC** | ***In-vitro* organ culture** |
| **LOS** | **Lipooligosaccharide** |
| **LPS** | **Lipopolysaccharide** |
| **µl** | **micro-Litre** |
| **µM** | **micro-Molar** |
| **MAPK** | **Mitogen Activated Protein Kinase** |
| **MH** | **Mueller-Hinton** |
| **min** | **Minute** |
| **ml** | **milli-Litre** |
| **mRNA** | **Messenger ribonucleic acid** |
| **MOI** | **Multiplicity of infection** |
| **NF-κB** | **Nuclear factor-κB** |
| **nM** | **nano-Molar** |
| **NOD** | **Nuclear-binding Oligomerisation Domain** |
| **OD** | **Optical density** |
| **PAGE** | **Poly-acrylamide Gel Electrophoresis** |
| **PAMP** | **Pathogen associated molecular pattern** |
| **PBS** | **Phosphate buffered saline** |
| **PCR** | **Polymerase chain reaction** |
| **PGN** | **Peptidoglycan** |
| **RNA** | **Ribonucleic acid** |
| **RPM** | **Round per minute** |
| **PRR** | **Pattern Recognition Receptor** |
| **RT** | **Room temperature** |
| **RT-PCR** | **Reverse transcriptase polymerase chain reaction** |

| | |
|---|---|
| **SDS** | **Sodium-Dodecyl-Sulphate** |
| **sec** | **Second** |
| **SEM** | **Scanning Electron Microscopy** |
| **+/-SEM** | **+/- Standard Error of Mean** |
| **si RNA** | **small interfering RNA** |
| **TBS** | **Tris buffered saline** |
| **TEER** | **Transepithelial Electrical Resistance** |
| **TLR** | **Toll-Like Receptor** |
| **TNFα** | **Tumour necrosis factor α** |
| **UC** | **Ulcerative colitis** |
| **VAIN** | **Variable atmosphere incubator** |
| **WT** | **Wild type** |

# CHAPTER 1

# Introduction

## 1.1 *Campylobacter jejuni*

### 1.1.1 Background

*Campylobacter* was first identified in 1906, when two British veterinary surgeons reported the presence of "large numbers of a peculiar organism" in the uterine mucous of pregnant sheep (McFadyean and Stockman, 1913; Skirrow, 2006). This was part of a major investigation into epizootic abortion in cattle and sheep initiated by the British government. The organism described was most likely *Campylobacter fetus* (subspecies *fetus*), a common cause of veterinary disease. Although uncommon in humans, *C. fetus* can cause septic disease in immunodeficient patients (Sauerwein *et al.*, 1993). In 1947, Vinzent and colleagues isolated this organism from the blood of three pregnant women admitted to hospital with sepsis, two of whom later aborted (Vincent *et al.*, 1947). *Campylobacter* were initially classified as *Vibrio spp.* due to spiral morphologies and it was not until 1963 that Sebald and Véron postulated the new genus *Campylobacter* (Sebald and Véron, 1963). The first report on *Campylobacter* enteritis dates back to 1938 when a milk-borne outbreak of diarrhoea was reported, now regarded as the first documented instance of human *Campylobacter* enteritis (Levy, 1946). Inability to culture the organism delayed identification of *Campylobacter jejuni* as a major cause of bacterial diarrhoea until the 1970s (Butzler *et al.*, 1973). Although significant progress has been made in the diagnosis and treatment of *Campylobacter*-related disease(s), the continuous problem of colonization of live-stock combined with antibiotic resistance adds certain urgency for a need to further our understanding of how this organism causes the observed clinical spectrum.

### 1.1.2 Taxonomy, typing methods and diversity

The group of *Campylobacteriaceae* include the genera *Campylobacter* and Arcobacter. Together with the closely related group of *Helicobacteriaceae* (*Helicobacter* and *Wolinella*) they form the delta-epsilon subdivision of the Proteobacteria (purple bacteria) (Gupta, 2000). Interestingly, many of these species are host-associated (*Helicobacter, Campylobacter, Wolinella*) and represent important human and animal pathogens (Eppinger *et al.*, 2004). Out of the known *Campylobacter species*, *Campylobacter jejuni* (*C. jejuni)* and *Campylobacter coli* (*C. coli*) are the most important human pathogens (Ketley, 1997). In addition, *Campylobacter upsaliensis* is being increasingly accepted as causative agent for diarrhoeal disease, particularly in children (Jimenez *et al.*, 1999). In contrast, *Campylobacter fetus spp.* and *Campylobacter venerealis* are mainly associated with disease in animals (Bawa *et al.*, 1991). Diversity of *C. jejuni* strains is striking with regards to both genotypic and phenotypic features. One possible explanation may be that *C. jejuni* cells are naturally able to take up DNA (Wang and Taylor, 1990; Wassenaar *et al.*, 1995) leading to significant genetic exchange when different *C. jejuni* strains are present. Various typing methods have allowed identification and further classification of different strains. Serotyping detects soluble heat-stable or heat-labile antigens which are exposed on the bacterial cell surface. The heat-labile typing scheme developed by Lior *et al.* recognizes over 100 different serotypes of *C. jejuni*, *C. coli* and *C. lari* (Lior *et al.*, 1982). Another serotyping scheme developed by Penner and colleagues was further modified by Frost *et al.*, and involves detection of heat-stable antigens by hemagglutination (Penner and Hennessy, 1980; Frost *et al.*, 1998). The heat-stabile antigen detected was originally thought to be a long chain lipopolysaccharide (LPS), however increasing evidence supported by genetic studies suggests that the serodeterminant antigen relates to structures within the capsular polysaccharide of *C. jejuni* (Karlyshev *et al.*, 2000).

Another phenotypic typing method developed for *Campylobacter* characterises isolates using bacteriophages. Phage-typing is currently used for surveillance in the UK (Frost *et al.*, 1999). Combination of serotype and phage type allows a greater level of discrimination of isolates. In addition to these phenotypic typing schemes there are multiple ways to demonstrate the genotypic diversity of *C. jejuni* strains. These include molecular-based approaches such as Polymerase Chain Reaction (PCR)-Deoxyribonucleic Acid (DNA) fingerprinting, DNA-DNA hybridization, multilocus sequence typing (MLST) and PCR-restriction fragment length polymorphism (RFLP) analysis (Johnsen *et al.*, 2006; Price *et al.*, 2006). Moreover, using DNA microarrays to compare whole genome of microbes (genomotyping) combined with computerised algorithms (i.e. Bayesian-based algorithms) allows the discrimination of strains from different sources (Champion *et al.*, 2005).

### 1.1.3 Transmission and epidemiology

*Campylobacter spp.* are part of the commensal flora in a wide range of domestic and wild animals including chicken, cattle and swine (Park, 2002). Transmission therefore occurs mainly *via* food, dairy products and contaminated water-based environmental sources (Ketley, 1997). *C. jejuni* represents the leading cause of acute bacterial enteritis world-wide. Incidence of infection, pattern of occurrence and mode of transmission differ significantly between countries and continents.

In several developed countries the number of reported *C. jejuni* cases currently exceeds 80 per 100,000 people (Friedman *et al.*, 2000). In England and Wales, 44,342 cases (~80 per 100,000) were reported in 2005 (Department for Environment Food and Rural Affairs, 2005), while average numbers in the US are lower with annual incidence of 21.9 cases per 100.000 (Samuel *et al.*, 2004). As *Campylobacter* enteritis rarely requires hospitalization, under-reporting is most likely such that the true incidence is likely to be

much higher (Tauxe, 1992; Friedman *et al.*, 2000). *Campylobacter* enteritis in industrialized countries is mainly due to sporadic infection transmitted *via* consumption of contaminated raw or undercooked meat (Deming *et al.*, 1987; Kapperud *et al.*, 1992). However, outbreaks do occur and are frequently associated with contaminated milk (Kalman *et al.*, 2000; Jimenez *et al.*, 2005). More recently, non live stock *C. jejuni* has been identified as major sources of human disease (Champion *et al.*, 2005). Infection shows a seasonal pattern with peaks during warmer months of the year, possibly associated with climatic conditions (Altekruse *et al.*, 1999; Padungton and Kaneene, 2003). Several case control studies have identified a number of major risk factors for acquiring infection in industrialized countries including handling or eating of raw or undercooked chicken, contact with pets or farm animals and travel abroad (Tauxe, 1992; Neal and Slack, 1997). All age groups can be affected, with one peak in children <4yrs, and a second peak in young adults (15-24yrs) (Tauxe, 1992; Friedman *et al.*, 2000). In developing countries *Campylobacter* is hyperendemic owing to poor sanitation and close contact with animals (Tauxe, 1992). Despite a lack of incidence data from national surveys, case-control community-based studies have provided estimates of 40,000 to 60,000/100,000 for children <5yrs of age (Oberhelman and Taylor, 2000; Rao *et al.*, 2001). Typically there is a decreasing infection-to-illness ratio, shortening of infection duration and by late childhood symptomatic infections are rare while asymptomatic carriage being frequently observed (Megraud *et al.*, 1990). An estimated incidence of 90/100,000 for the general population further confirms that *C. jejuni* infection in developing countries is mainly a paediatric disease (Tauxe, 1992; Rao *et al.*, 2001).

### 1.1.4 Clinical manifestations

The most common *Campylobacter* strains causing human disease are *C. jejuni* and *C. coli* (Lastovica and Skirrow, 2000). Although infection typically causes

gastroenteritis/enterocolitis, the clinical picture varies significantly in duration, severity and associated symptoms (Ketley, 1997). Furthermore, despite being self-limiting in the majority, post-infectious complications can be severe, and occasionally life threatening. The spectrum of disease associated with *C. jejuni* is summarized in Table 1.1.

1.1.4.1 Intestinal manifestations

The most common manifestation of *C. jejuni* infection is acute gastroenteritis. Following an incubation period of ~24-72h, symptoms develop including cramping abdominal pain which can sometimes be associated with fever, vomiting and headaches (Allos, 2001). Pain can be generalized or localized, the latter case sometimes difficult to distinguish from acute appendicitis (Blakelock and Beasley, 2003). Diarrhoea typically develops shortly after onset of abdominal pain and can vary significantly amongst individuals, i.e. from a mild non-inflammatory watery presentation to severe and bloody (Blaser, 1997). Sigmoidoscopy if performed, usually reveals mucosal changes ranging from oedema and hyperaemia, petechial haemorrhages and mucosal friability. Inflammation can also be observed in parts of the ileum and jejunum commonly associated with mesenteric adenitis (Ketley, 1997; Wassenaar and Blaser, 1999).

Infection typically lasts for ~7 days in an otherwise healthy, immunocompetent individual. One may hypothesize that disease outcome is likely to be dependent on both the virulence of the infecting strain and the host immune status. Intriguingly, in developing countries where symptomatic infection is restricted to young children, clinical presentation tends to be mild, with watery, non inflammatory diarrhoea (Blaser, 1997; Oberhelman and Taylor, 2000). In contrast, in the developed world, infected individuals generally suffer severe symptoms with more frequent, bloody, inflammatory diarrhoea. Importantly, patients acquiring infections abroad typically present clinical features of their country of origin, thus highlighting a major impact of host immune status on disease pathogenesis (Oberhelman and Taylor, 2000). As a post-infectious

intestinal manifestation, irritable bowel syndrome (IBS) has been increasingly linked to enteric *C. jejuni* infection. IBS is characterised by abdominal pain sometimes associated with altered bowel habit following bacterial enteritis (Spiller, 2007). Various other intestinal complications have been reported and are summarized in Table 1.1.

| **Spectrum of disease caused by *Campylobacter spp.*** |
|---|
| **Gastrointestinal** |
| • **Enteritis**, (Crushell *et al.*, 2004)<br>• **Appendicitis**, (Campbell *et al.*, 2006)<br>• **Irritable bowel syndrome**, (Spiller, 2007)<br>• **Toxic megacolon and perforation**, (Larvol *et al.*, 1994)<br>• **Hepatitis**, (Korman *et al.*, 1997)<br>• **Pancreatitis**, (Kandula *et al.*, 2006)<br>• **Cholecystitis**, (Dakdouki *et al.*, 2003)<br>• **Peritonitis**, (Calonge Raventos *et al.*, 2002)<br>• **Splenic rupture**, (Frizelle and Rietveld, 1994) |
| **Rheumatological** |
| • **Reactive arthritis**, (Hannu *et al.*, 2004)<br>• **Reiters Syndrome**, (Peterson, 1994)<br>• **Septic arthritis**, (Cone *et al.*, 2003)<br>• **Henoch-Schönlein purpura**, (Apostolopoulos *et al.*, 1999) |
| **Neurological** |
| • **Guillain-Barré syndrome**, (Kuwabara, 2007)<br>• **Miller-Fischer syndrome**, (Godschalk *et al.*, 2007)<br>• **Brainstem encephalitis**, (Hussain *et al.*, 2007)<br>• **Meningoencephalitis**, (Tsugawa *et al.*, 2004) |
| **Skin** |
| • **Cellulitis**, (Monselise *et al.*, 2004) |
| **Lung** |
| • **Pneumonia**, (Sakran *et al.*, 1999) |
| **Intravascular** |
| • **Septic shock**, (Meyrieux *et al.*, 1996)<br>• **Endocarditis**, (Sitter *et al.*, 1992)<br>• **Myocarditis**, (Pena and Fishbein, 2007)<br>• **Thrombophlebitis**, (Ozaki *et al.*, 1999)<br>• **Hemolytic uremic syndrome**, (Dolezel *et al.*, 1993)<br>• **Aneurysm**, (Grollier *et al.*, 1993) |

**Table 1.1: Spectrum of reported disease caused by *Campylobacter spp.***

#### 1.1.4.2 Extraintestinal manifestations

Overall, incidence of extraintestinal manifestations associated with *C. jejuni* infection is low compared to enteric disease. However, when they do occur they can be severe and potentially live threatening. To date the most extensively studied is Guillain-Barré syndrome (GBS), an acute post-infectious ascending paralysis that can affect peripheral and cranial nerves (particularly VII, facial nerve), in severe cases requiring artificial ventilation (Kuwabara, 2007). *C. jejuni* is now recognised as the most common identifiable pathogen associated with development of GBS. Molecular mimicry of the peripheral nerve gangliosides *by C. jejuni* lipooligosaccharide (LOS) results in the generation of auto-reactive antibodies causing inflammation and tissue damage (Komagamine and Yuki, 2006; Godschalk *et al.*, 2007). Several other conditions have been reported in association with *C. jejuni* infection including pneumonia, hepatitis, nephritis, reactive arthritis and myocarditis but are mostly limited to few reported cases (Table 1.1). In contrast to otherwise healthy individuals, immunocompromised patients such as human immunodeficiency virus (HIV) positive individuals, generally suffer from more severe, prolonged and/or relapsing illness. Furthermore, occurrence of extraintestinal manifestations is more frequent, most likely due to a higher incidence of bacteraemia in these patients (Pigrau *et al.*, 1996; Manfredi *et al.*, 1999; Crushell *et al.*, 2004). Recently an association between *C. jejuni* infection and immunoproliferative disease of the small intestine has been reported (Lecuit *et al.*, 2004).

### 1.1.5 Antibiotic resistance

Although *C. jejuni* infection is self-limiting, antibiotics are recommended to those with severe and/or prolonged enteritis, septicaemia or extraintestinal complications. For confirmed *C. jejuni* infection, the macrolide antibiotic erythromycin is the first choice of treatment (Williams *et al.*, 1989). Fluoroquinolones (ciprofloxacin) are also used in

enteritis of unknown origin, particularly in suspected travellers' diarrhoea (Al-Abri *et al.*, 2005). Resistance to both macrolides and fluoroquinolones has been increasing amongst *Campylobacter* species (Engberg *et al.*, 2001; Gibreel and Taylor, 2006). This is most likely due to persistent antibiotic use in livestock (Angulo *et al.*, 2004). Amplification and geographical spread of strains (clonal dissemination) might also contribute to the observed increase in *Campylobacter* resistance (Bae *et al.*, 2007). Induction of antibiotic resistance in humans is well recognised particularly for the use of fluroquinolones (Engberg *et al.*, 2004). However, with human-to human transmission being rare in *Campylobacter* infection, the overall effect of this mechanism to resistance development is probably minimal.

In recent years, greater insight into the development of antibiotic resistance of *Campylobacter* has been gained, hopefully opening up potential for new strategies to combat resistant strains. *Campylobacter* is known to express two multidrug efflux pump systems CmeABC and CmeDEF, and evidence suggests a role in mediating resistance to several antibiotics (Akiba *et al.*, 2006; Quinn *et al.*, 2006). For example, specific inhibition of CmeABC by an efflux pump inhibitor can decrease the Minimal Inhibitory Concentration (MIH) of erythromycin resistant strains and increase the effect of various antibiotics, suggesting a potentially promising approach in preventing and treating antibiotic resistance of *Campylobacter* in humans and animal reservoirs (Martinez and Lin, 2006). Furthermore, mutagenesis of genes coding for antibiotic target sites such as 23S rRNA as target site for macrolide antibiotics and gyrase (i.e. gyrA and parC), for fluoroquinolones are well recognised in *Campylobacter* strains resistant to the corresponding antibiotic (Engberg *et al.*, 2001; Gibreel and Taylor, 2006).

### 1.1.6 *C. jejuni* disease pathogenesis

*C. jejuni* colonises warm blooded animals and birds without causing disease. However, in humans, ingestion of a relatively low dose can cause a wide range of pathology (Black *et al.*, 1988). Despite the serious health problem caused by the bacterium disease pathogenesis remains poorly defined. Bacterial adhesion and invasion of the intestinal epithelium is a well-established early event prior to initiation of inflammatory processes and diarrhoeal development. The "invasiveness" of *C. jejuni* strains is often used as a measure for bacterial virulence reflecting the involvement of multiple bacterial structures and mechanism(s) in this process. In the following section, bacterial factors and mechanism(s) implicated in *C. jejuni* disease pathogenesis are described.

#### 1.1.6.1 General microbiology of *C. jejuni*

*Campylobacter* (Greek "curved rod") is a Gram-negative, spirally shaped organism measuring 1.5-6 μm in length and 0.2-0.5 μm in width (Figure 1.1). The bacterium contains a polar flagellum at one or both ends leading to a high degree of motility and the typical darting and spinning movements observed under the microscope. For ideal growth, *Campylobacter* requires a microaerophilic environment with $O_2$ concentrations of 3-15% and $CO_2$ levels of 3-5%. The optimal growth temperature lies between 37-42°C most likely reflecting adaptation to its natural habitat, i.e. the intestine of warm-blooded animals and birds (Ketley, 1997).

#### 1.1.6.2 Lipooligosaccharide (LOS)

Lipopolysaccharide (LPS) and LOS are major constituents of the outer membrane of Gram-negative bacteria and play a critical role in the pathogenesis of a variety of bacterial infections (Jacques, 1996; Preston *et al.*, 1996). LPS and LOS contribute to serum resistance, antibiotic resistance and endotoxicity to pathogens. LPS consists of three major components: lipid A, core oligosaccharide, and an O-chain consisting of oligosaccharide units.

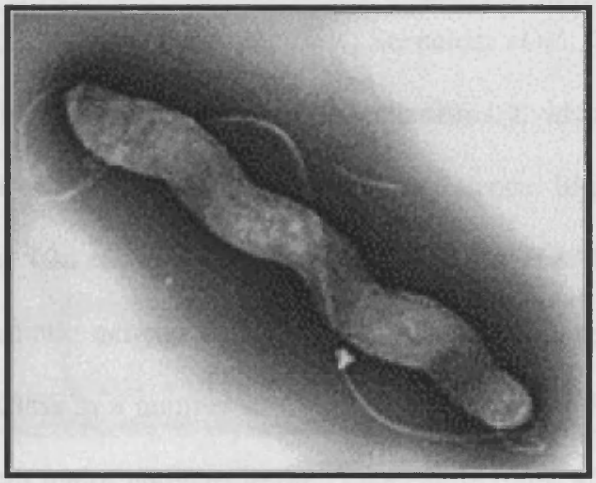

**Figure 1.1: Scanning Electron Micrograph of *Campylobacter jejuni*.** The spiral shape and bipolar flagella are illustrated (from Homepage of the Department of Tropical and Infectious Diseases, LHTM, London, UK).

LOS lacks the O-chain, with only one oligosaccharide unit present (Moran *et al.*, 1996). Until the publication of the whole genome sequence, *C. jejuni* was believed to produce either LPS or LOS. However, repeating oligosaccharide units once thought to be an LPS-associated O-chain is now considered to be capsular in origin (Karlyshev *et al.*, 2000). Antigenic mimicry of human gangliosides by *C. jejuni* LOS contributes to the development of GBS (Goodyear *et al.*, 1999; Komagamine and Yuki, 2006). Moreover, levels of LOS phase variation seems to be associated with serological responses of *C. jejuni* and the capacity of ganglioside mimicry (Guerry *et al.*, 2002; Prendergast *et al.*, 2004).

1.1.6.3 Capsular polysaccharide (CPS)

CPSs are a common feature on bacterial surfaces, known to play an important role in bacterial survival, persistence in the environment and often contribute to disease pathogenesis (Roberts, 1996; Watts *et al.*, 2005). They can exhibit structural variation, mimicry to host antigens, resistance to phagocytosis and complement-mediated killing (Moe and Granoff, 2001; Segura *et al.*, 2004; Schneider *et al.*, 2007). Thus CPSs allow bacteria to evade host immunity *via* several mechanisms. Evidence for the production of CPSs by *C. jejuni* was suggested by its genome sequence. Identification of *kps* genes potentially involved in capsule biosynthesis of NCTC11168 (Karlyshev *et al.*, 1999) prompted a systematic genetic analysis of the corresponding locus and resulted in identification of CPSs in a number of *C. jejuni* strains (Karlyshev *et al.*, 2000). These molecules represent major antigens in the Penner serotyping scheme (Karlyshev *et al.*, 2000). Studies performed on *C. jejuni* 81-176 suggested a role for the capsule in serum resistance, epithelial invasion and diarrhoeal disease (Bacon *et al.*, 2001). Subsequent characterisation (Karlyshev and Wren, 2001) led to visualisation of the capsule by electron microscopy (Figure 1.2) (Karlyshev *et al.*, 2001). These experiments suggested that the previously described high molecular weight "lipopolysaccharides" (HMW LPSs) of *C. jejuni* are in fact CPSs. A role for *C. jejuni* CPS in disease pathogenesis was suggested as a capsule-deficient 81-176 mutant strain shows reduced invasion and virulence *in vitro* and in a ferret diarrhoeal model (Bacon *et al.*, 2001). Another study utilising a capsule-deficient mutant of parental strain 81116 also noted reduced adherence to human intestinal epithelia, in contrast no significant difference in colonization of the chicken gut was found between wild-type and mutant, suggesting contribution of bacterial CPS to host-pathogen interactions maybe species dependent (Bachtiar *et al.*, 2007).

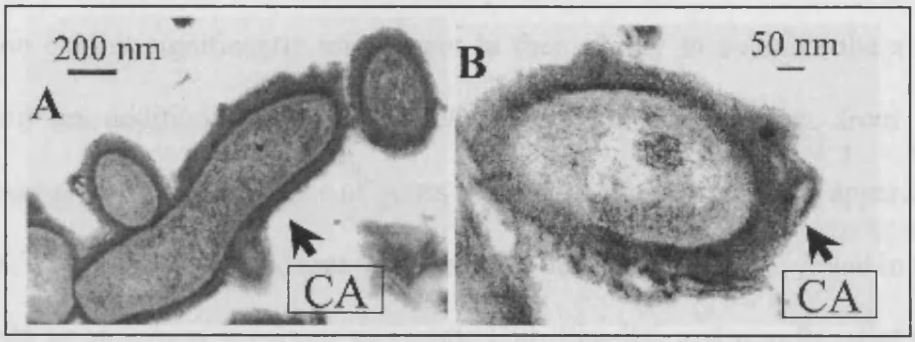


**Figure 1.2: *C. jejuni* capsular polysaccharide (CPS).** Transmission electron microscopy of *C. jejuni* stained with Alcian blue at two magnifications. CA indicates capsular polysaccharide (from Karlyshev *et al.* ,2001).

1.1.6.4 Flagellin

The basic structure of bacterial flagellum consists of a basal body, which is embedded in the cell envelope and connected to the filament *via* a universal joint, the torsion hook. The flagellar filament consists of several thousands of self-assembling protein (flagellin) monomers arranged in a helix (Macnab, 2003). The major *C. jejuni* flagellar filament subunits are Flagellin A (FlaA) and Flagellin B (FlaB); their encoding genes *flaA* and *flaB* are arranged head-to-tail on the genome and show >90% sequence homology (Guerry *et al.*, 1990).

*Campylobacter* contains a single unsheathed flagellum at one or both poles providing the organism with a high degree of motility (Figure 1.1). This is necessary to overcome peristalsis and entry into the mucous layer. *C. jejuni* flagellum-mediated motility is a prerequisite in both human disease and in successful colonisation in animals (Wassenaar *et al.*, 1993). An association between the presence of intact flagellum and ability of *C. jejuni* to adhere and invade has been reported (Wassenaar *et al.*, 1991; Grant *et al.*, 1993; Nachamkin *et al.*, 1993; Yao *et al.*, 1994). Mutants with reduced motility but

intact FlaA expression colonise chickens to a similar extent as the parental strain (Wassenaar *et al.*, 1993). In contrast, fully motile mutants but deficient in FlaA expression exhibit significantly impairment in their ability to colonize the avian gut, suggesting an additional role for flagellum in virulence, distinct from motility (Wassenaar *et al.*, 1993). Absence of genes encoding a type III secretion apparatus in *C. jejuni* NCTC11168 led two independent groups to demonstrate that *C. jejuni* in fact uses its flagella as an export apparatus to secrete both flagellar and non-flagellar proteins, some of which involved in adhesion and invasion (Konkel *et al.*, 2004; Song *et al.*, 2004). *C. jejuni* flagellin is also decorated by glycostructures that are antigenic, this will be discussed later.

1.1.6.5 Cytolethal distending toxin (CDT)

CDT is produced by several Gram-negative bacteria including *E. coli*, *Salmonella*, *Haemophilus ducreyi* and *Helicobacter spp.* (Johnson and Lior, 1988b; Okuda *et al.*, 1995; Cope *et al.*, 1997). CDT was first identified as a component of *C. jejuni* in 1987, since then considerable progress has been made in elucidating its role in disease pathogenesis (Johnson and Lior, 1988a). Briefly, CDT causes eukaryotic cells to arrest in the G2/M phase of cell-cycle, preventing them from entering mitosis leading to cell death. The toxin was named after the morphological changes associated with its action; cytoplasmic distension. CDT consists of three subunits (CdtA, B and C) encoded by three adjacent or slightly overlapping open reading frames (ORFs) assigned *cdtA*BC (Ceelen *et al.*, 2006). CdtB is the active subunit sharing homology with mammalian DNase I (Lara-Tejero and Galan, 2000). In order to function as a DNase CdtB needs to gain access to the nuclear compartment and it is suggested that CdtA & C may promote delivery of CdtB into host cell (Lara-Tejero and Galan, 2001). This hypothesis is supported by Lee and colleagues who demonstrated binding of CdtA and CdtC, but not CdtB to HeLa cells (Lee *et al.*, 2003).

All three CDT proteins are membrane-associated and required for the induction of pro-inflammatory cytokine IL-8 (Hickey *et al.*, 2000). The same authors also suggest a role for CdtC in mediating or enhancing cytotoxicity. Utilising a Nuclear factor-κB (NF-κB) deficient murine infection model, Fox and colleagues confirmed a role for CDT in eliciting inflammatory responses *in vivo* (Fox *et al.*, 2004).

1.1.6.6 Glycosylation systems

The function of post-translational glycosylation in eukaryotic cells is manifold (Rudd *et al.*, 2001). Only recently, the long accepted dogma that bacteria only express non-glycosylated proteins has been disproved conclusively, opening the door to new insights into prokaryotic protein structure and function (Schmidt *et al.*, 2003).

As early as 1989, Logan and colleagues provided clear evidence that *Campylobacter* flagellin was post-translationally modified (Logan *et al.*, 1989). Following their observation, studies began to unravel new aspects of *Campylobacter* glycosylation. Today we know that *Campylobacter* is almost unique as it contains both a general *N*-linked protein glycosylation pathway (responsible for post-translational modification of at least 30 proteins) as well as an *O*-linked system (responsible for flagellar gylcosylation) (Szymanski *et al.*, 2003; Karlyshev *et al.*, 2005a; Szymanski and Wren, 2005).

The *N*-linked glycosylation machinery of *C. jejuni* is encoded by a single gene cluster named *pgl* (protein glycosylation) (Szymanski *et al.*, 1999). The key enzyme in the general glycosylation locus is PglB, identified as the first example of a bacterial *N*-linked oligosaccharyltransferase. Functionality of this enzyme was elegantly demonstrated by Wacker *et al.* who transferred the *pgl* locus into *E. coli* and monitored effective glycosylation of a co-expressed *C. jejuni* protein AcrA (Wacker *et al.*, 2002).

Studies investigating the biological role for *N*-linked glycosylation clearly link it to bacterial virulence. An isogenic 81116 *pglH* mutant is deficient in its ability to

glycosylate a number of proteins and showed reduced ability to adhere/invade human epithelia and colonize chicks (Karlyshev *et al.*, 2004). Kakuda and DiRita tested 22 different glycosylation mutants in strain 81-176, one of which, Cj1496c was found to influence invasion of human epithelium cells and colonisation of the avian gut (Kakuda and DiRita, 2006). Studies have shown *O*-linked glycosylation is essential for successful flagellin assembly and motility hence influencing adhesion, invasion and virulence *in-vivo* (Thibault *et al.*, 2001; Guerry *et al.*, 2006). In contrast to the *N*-linked system, the genetic locus of the *O*-linked system is more heterogeneous and genetically diverse (Parkhill *et al.*, 2000a; Champion *et al.*, 2005). A predominant mechanism for this variability is the presence of seven contingency or phase variable genes in the flagellin glycosylation locus that can contribute to antigenic variation (Karlyshev *et al.*, 2005a). Such genes, also found in the capsule locus, enable rapid reversible expression of genes by slip strand mispairing (Karlyshev *et al.*, 2005b). This can be considered as a simple primordial mechanism used by bacteria for variable gene expression. It is tempting to speculate that *Campylobacter* flagellin glycostructure and CPS determinants undergo these changes in response to pressures exerted by the host. However, this is not proven and it could be due to other evolutionary pressures such as avoidance of bacteriophage that also frequent the natural habitat of *C. jejuni* (i.e. avian gut).

1.1.6.7 Chemotaxis, adhesion and invasion

Some evidence suggest that in addition to motility, chemotaxis is important for intestinal colonisation by *C. jejuni* (Takata *et al.*, 1992; Hendrixson *et al.*, 2001). A number of genes involved in motility other than flagellin have been identified, including *cheY*, *cetA*, and *cetB* (Yao *et al.*, 1994; Hendrixson *et al.*, 2001; Golden and Acheson, 2002).

Once motility and chemotaxis allow *C. jejuni* to reach the surface of the intestinal epithelium, adherence has to be established as a prerequisite for cell invasion. Unlike invasion, intestinal colonization can be established in the mucous layer without the need for adherence (Lee *et al.*, 2005). In addition to factors mediating *C. jejuni* adherence and invasion mentioned above, specific molecules, named adhesins are also directly involved in this process. Adhesins are located on the bacterial surface and interact with corresponding receptors on the host cell surface to initiate adhesion. Several *C. jejuni* adhesins have been identified including the outer membrane proteins CadF (a fibronectin binding protein) (Monteville *et al.*, 2003), PEB1 (a homolog of Gram-negative ABC transport system) (Pei and Blaser, 1993), JlpA (surface lipoprotein, Jejuni lipoproteinA) (Jin *et al.*, 2003), major outer membrane protein (MOP) (Moser *et al.*, 1997), the autotransporter protein CapA (Ashgar *et al.*, 2007) and LOS (McSweegan and Walker, 1986). In contrast, only few receptors on host intestinal epithelia that interact with *C. jejuni* are known. CadF has been shown to interact with Fibronectin (Fn) not only leading to adherence but also initiating host signalling associated with bacterial uptake (Monteville and Konkel, 2002). Bacterial JlpA can bind to the eukaryotic heat shock protein (HsP) 90, leading to activation of host signalling events (Jin *et al.*, 2003). As for other invasive enteropathogens, rearrangement of host cytoskeletal structures, i.e. microtubules (MT) and microfilaments (MF) have been suggested to mediate internalisation of *C. jejuni* (Biswas *et al.*, 2003). Additionally, more recently involvement of host cell small GTPases (Rac1 and Cdc42) in bacterial internalization has been suggested (Krause-Gruszczynska *et al.*, 2007). *C. jejuni* preferentially enters host cells *via* the basolateral surface of polarized epithelia while entry *via* the apical surface seems to be less efficient (Monteville and Konkel, 2002). Whether *C. jejuni* translocates to the basolateral surface *via* a paracellular route (migration between cells) or a transcellular route (migration via M-cells, followed by

intracellular trafficking) remains unclear (Figure 1.3) (Kopecko *et al.*, 2001a). However, there is no doubt that host bacterial cross-talk is intimately linked to activation of innate and adaptive immune responses leading to inflammation, diarrhoea and ultimately clearance of the infective agent.

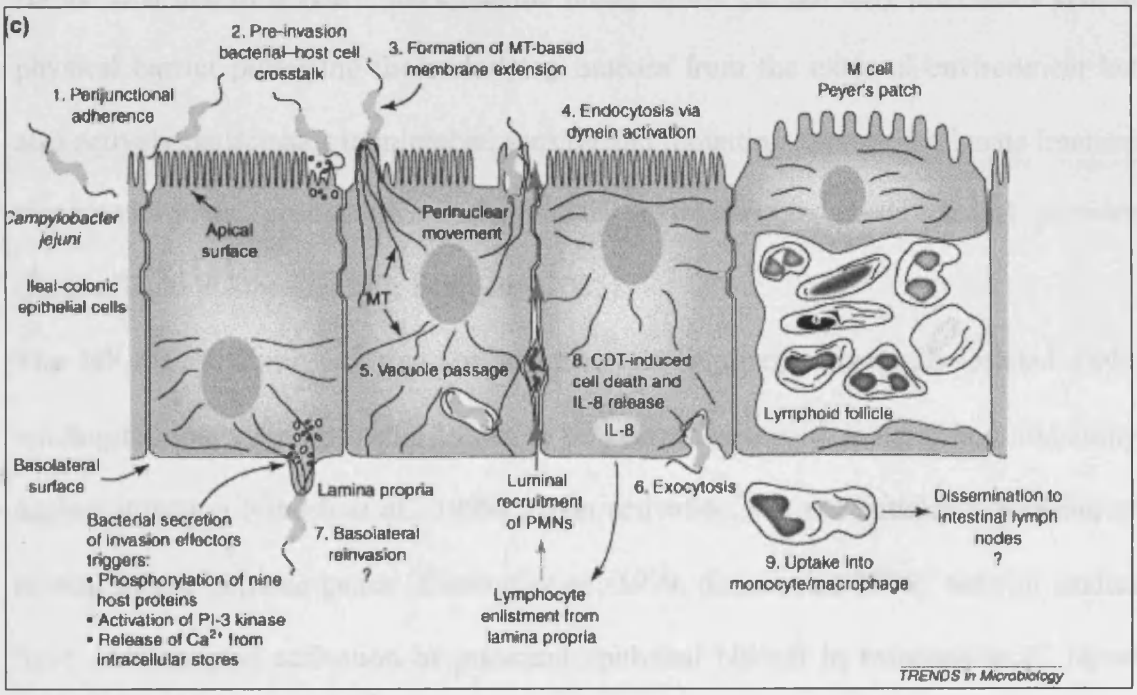


**Figure 1.3: Interaction of *C. jejuni* with polarised intestinal epithelia.** Multiple host bacterial interactions are involved in *C. jejuni* adhesion and invasion of polarised epithelia leading to activation of immune responses (from Kopecko *et al.*, 2001).

### 1.1.7 Host response

The variable clinical picture of *C. jejuni* enteritis and lack of direct correlation with strain virulence underlines the crucial role of host immune response(s) in determining disease severity and outcome. Furthermore, given the generally self-limiting nature of infection, insight into immune defence mechanism(s) that allow successful bacterial clearance is essential to further our understanding of *C. jejuni* disease pathogenesis.

1.1.7.1 Innate host response

a) Intestinal epithelium

As the first line of defence, the epithelial lining of the gut not only provides a crucial physical barrier protecting the underlying mucosa from the external environment but also actively participates in microbial sensing and mounting appropriate innate immune responses by the production of chemokines, cytokines and antimicrobial peptides (Kagnoff and Eckmann, 1997; Eckmann, 2005).

The NF-κB/rel family of transcription factors comprises structurally related DNA binding/transactivation proteins known to be central regulators in early host immunity against infection (Ghosh *et al.*, 1998). Upon activation, NF-κB initiates expression of several innate immune genes (Elewaut *et al.*, 1999; Sansonetti, 2006). Several studies have demonstrated activation of intestinal epithelial NF-κB in response to *C. jejuni* infection (Mellits *et al.*, 2002; Jin *et al.*, 2003; Chen *et al.*, 2006; Johanesen and Dwinell, 2006). *C. jejuni* surface protein JlpA interacts with epithelial Hsp90 promoting bacterial adhesion leading to NF-κB and p38MAP kinase activation (Jin *et al.*, 2003). The cytokine Interleukin (IL)-8 is a potent chemo-attractant known to recruit immune cells including neutrophils to the site of local inflammation. Release of IL-8 in response to bacterial infection is thought to be a critical event in diarrhoea development and clearing of infection. *C. jejuni*-mediated induction and release of intestinal epithelial IL-8 is well established, to date bacterial adhesion/invasion (Hickey *et al.*, 1999) and the

presence of CDT (Hickey *et al.*, 2000) have been shown to be critical mediators of IL-8 production. The degree of IL-8 induction varies in *in-vitro* models of infection as not surprisingly, the response is dependent on both bacterial strain and cell-line employed (MacCallum *et al.*, 2006). Progress has been made in delineating signal transduction events that lead to IL-8 production during *C. jejuni* infection. Both ERK and p38 MAP kinases are involved in *C. jejuni*-mediated host responses with the ERK pathway critical for IL-8 induction (MacCallum *et al.*, 2005; Watson and Galan, 2005). Prior to activation of MAP kinases and NF-κB, host sensing of bacteria and/or bacterial components is required. In principal this interaction can be fulfilled by host pattern recognition receptors (PRRs) known to interact with conserved bacterial motifs called pathogen-associated molecular patterns (PAMPs) (Akira *et al.*, 2006; Sanderson and Walker, 2007). The most extensively studied family of PRRs are the Toll-like receptors (TLRs) with TLR5 expressed at the basolateral surface of the intestinal epithelium (Gewirtz *et al.*, 2001b). TLR5 recognises flagellin of enteropathogens such as *Salmonella*, leading to activation of NF-κB (Hayashi *et al.*, 2001). Recent studies suggest *C. jejuni* flagellin to be a poor activator for epithelial TLR5. (Andersen-Nissen *et al.*, 2005; Watson and Galan, 2005; Johanesen and Dwinell, 2006). This seems to be due to differences in amino acid sequences of *C. jejuni* flagellin known to bind to TLR5 (Andersen-Nissen *et al.*, 2005). At present the role of other PRRs in eliciting host responses during *C. jejuni* infection remains unknown.

b) Other innate immune cells

Once *C. jejuni* has breached the intestinal epithelium cell (IEC) layer, the bacterium will encounter the underlying lymphoid tissue harbouring various other immune cells including macrophages, lymphocytes and dendritic cells (DCs). Several studies have demonstrated that *C. jejuni* is able to survive phagocytosis by human mononuclear cells, induce secretion of chemokines, and mediate apoptosis (Kiehlbauch *et al.*, 1985; Jones

*et al.*, 2003; Hickey *et al.*, 2005). Importantly, prolonged intracellular survival of *C. jejuni* for up to 7 days combined with bacterial replication might favour dissemination within the host (Hickey *et al.*, 2005). DCs are capable of traversing the tight junctions of the intestinal mucosa allowing them to interact directly with luminal bacteria (Niess and Reinecker, 2005). They have been shown to play a major role during infection with eneropathogens such as *E. coli* (Torres *et al.*, 2006), *Salmonella spp.* (Bueno *et al.*, 2005), both *via* antigen presentation and cytokine production (Niess and Reinecker, 2006). Activation of NF-κB and production of several cytokines including IL-1β, IL-6, IL-8, IL-10 and IL-12, INF-γ and TNF-α by DCs in the presence of *C. jejuni* has been noted. High IL-12 production with low IL-10 levels suggests *C. jejuni* initiates Th1-polarized immunity (Hu *et al.*, 2006b). At present, our understanding of DC activation in *C. jejuni*-mediated disease remains limited.

1.1.7.2 Adaptive immune response

a) Antibody response

Infection with *C. jejuni* is known to correlate with an increase in serum titres of specific IgG, IgM, and IgA antibodies (Blaser, 1997; Strid *et al.*, 2001). While IgM and IgA levels quickly decrease after infection, IgG antibodies can remain elevated for months or even years (Strid *et al.*, 2001). Re-challenge of previously infected adults who had proven serum antibody response were found to be protected from illness but not colonization, suggesting a role for humoral immunity in disease outcome (Black *et al.*, 1988). Furthermore, in developing countries recurrent infections result in an age-related rise in specific serum-antibodies, leading to successively milder symptoms with each infection (Taylor *et al.*, 1993). Immunocompromised patients, specifically those deficient in immunoglobulin production, suffer from more severe, prolonged and relapsing illness lending further support for the involvement of humoral immunity in

disease outcome (Coker *et al.*, 2002). Taken together, these findings suggest an important role for the humoral/antibody response in *C. jejuni* infection.

b) Cellular response

To date we have limited information on the role of cellular adaptive immune responses to *C. jejuni*. However, the established association of GBS with *C. jejuni* suggests that activation of T cells in response to infection can occur. In addition to cross-reactive antibodies, T lymphocytic infiltrations in affected nerve tissue and activated T cells in the blood from GBS patients have been demonstrated (Van den Berg *et al.*, 1995; Schmidt *et al.*, 1996). A T cell line established from a nerve biopsy of a GBS patient with a preceding *C. jejuni* infection was shown to consist entirely of T cells expressing the γδ-TCR (Ben-Smith *et al.*, 1996). The same authors also observed expansion of human γδ-T cells after stimulation of Peripheral Blood Mononuclear Cells (PBMCs) with *C. jejuni* (Ben-Smith *et al.*, 1997). However, the role, if any, of T cells in acute intestinal *C. jejuni* infection remains unknown.

## 1.2 Defensins: a family of antimicrobial peptides

Antimicrobial peptides (AMPs) are part of an ancient defence system of all forms of life. To date hundreds of AMPs have been isolated from single-celled organisms (e.g. bacteria), insects, plants, birds, fish and mammals including humans (Lehrer, 2007). AMPs are generally small size (12 to 100 amino acids), positively charged (cationic) and amphiphilic in nature (Ganz, 2003; Selsted and Ouellette, 2005). In addition to their antimicrobial properties, AMPs can also function as multieffector molecules capable of enhancing phagocytosis, stimulate prostaglandin release, neutralize septic effects of LPS and recruit various immune cells to the site of infection (Lehrer, 2007). In humans two major subgroups of cationic AMPs are found: defensins and cathelicidins. Both are structurally and evolutionarily distinct with similar distribution in human tissues,

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predominantly the epithelium and circulating phagocytes. In other mammals AMPs such as histatins (Devine, 2003), dermicindin (Schittek *et al.*, 2001) and anionic peptides (Brogden *et al.*, 1997) have been found. However, these are restricted to a few animal species and tissues.

In the following section, gene- and protein-structure, tissue distribution, regulation and function of human defensins are described with a focus on the gastrointestinal (GI) tract.

### 1.2.1 Gene and protein structure

Defensins are defined as an evolutionary conserved, gene-encoded family of vertebrate AMPs. They are between 38 and 44 amino acids long and contain a framework of six disulphide-linked cysteines (Figure 1.4) (Selsted and Ouellette, 2005). Like most other AMPs, defensins are cationic in nature (net charge ranging from +1 to +11) mainly due to the high percentage of arginine residues. Three defensin subfamilies are found in vertebrates: α-defensins, β-defensins and θ-defensins. In humans, however only α-defensins and β-defensins are expressed while θ-defensin-expression is restricted to rhesus monkey leucocytes (Tang *et al.*, 1999). Structurally, α- and β-defensins differ in the length of peptide segments and their di-sulphide pairing (Figure 1.4), however both adapt an antiparallel beta-sheet ("defensin fold") structure. In β-defensins, the β-sheet is flanked by an N-terminal α-helical segment of variable length (Hoover *et al.*, 2001).

The main defensin gene locus coding for all α-defensins and several β-defensins is located on a cluster on the short arm of chromosome 8 (8p22-23.1) near the telomere region (Harder *et al.*, 1997b; Liu *et al.*, 1997). The α-genes are flanked by the β-genes, the close proximity suggesting a common ancestral progenitor. Three additional β-defensin gene clusters within chromosomes 6 (6p12) and 20 (20q11.1 and 20p13) have been identified with comprehensive searches revealing up to 40 potential β-defensin

sequences (Schutte *et al.*, 2002; Patil *et al.*, 2005). Evidence suggests rapid evolution of mammalian defensin genes through duplication and diversification, a likely evolutionary response to changes in microbial exposure (Lynn *et al.*, 2004; Semple *et al.*, 2006). The genomic organization consists of two short exons separated by a large intron. Exon I encodes for the prepro peptide, consisting of an N-terminal hydrophobic, leucine rich signal sequence. Exon II encodes an anionic pro-segment and a C-terminal cationic segment that on cleavage yields the active peptide (Figure 1.4) (Lehrer and Ganz, 2002; Semple *et al.*, 2006). Neutrophil defensins (HNP1-4) have a short extra exon that encodes the leading peptide, Exon I and II encode for an untranslated region and the preproregion, respectively (Semple *et al.*, 2006).

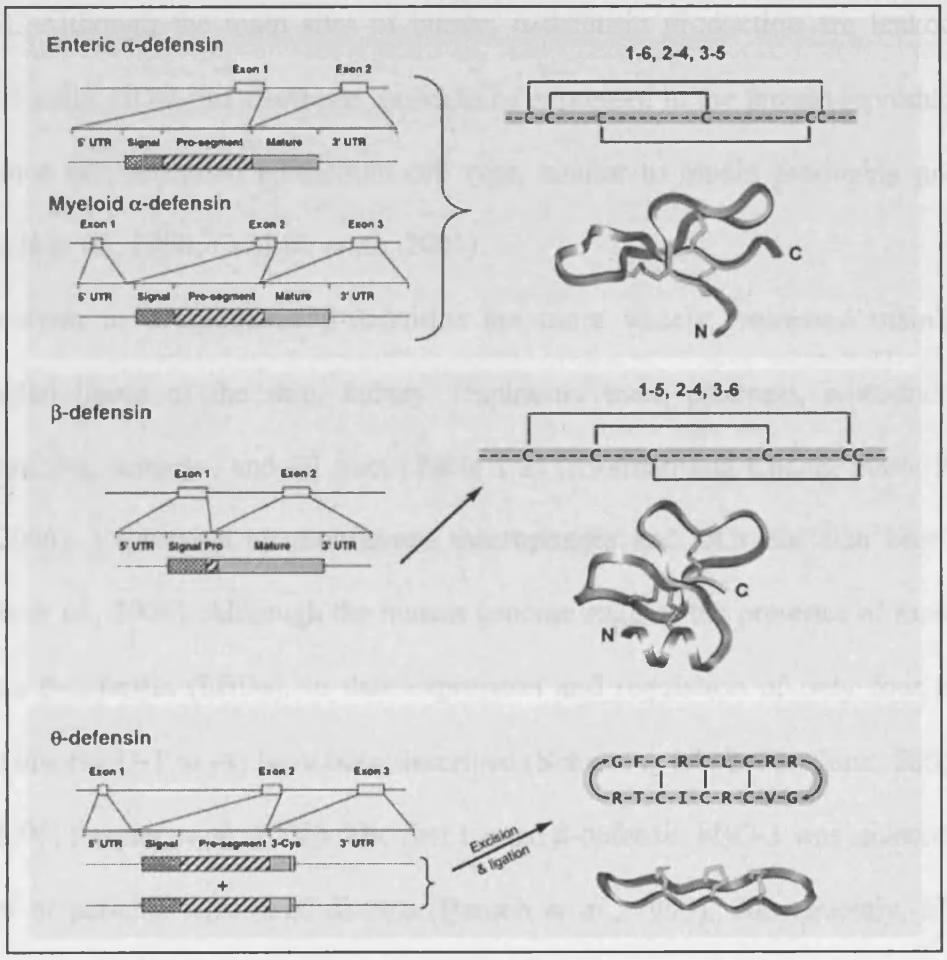



**Figure 1.4: Defensin gene and peptide structure (from Selsted and Ouellette, 2005).**

### 1.2.2 Tissue distribution

Defensins are expressed in various cells and tissues that are exposed to microbes (Table1.2). Human α-defensins 1-4 are predominantly expressed in promyelocytes (precursor cells derived from bone marrow) and stored in primary, azurophilic granules of neutrophils (Ganz *et al.*, 1985; Ganz, 1987). They are therefore also referred to as human neutrophil peptides (HNPs).

More recently it has been shown that monocytes, natural killer (NK) cells and T cells also express HNPs (Table 1.2) (Agerberth *et al.*, 2000; Chalifour *et al.*, 2004). Human α-defensins HD-5 and HD-6 are expressed in Paneth cells, which are located in the crypts of Liberkühn throughout the small intestine (Jones and Bevins, 1992; Ouellette, 2006). Although the main sites of human α-defensin production are leukocytes and Paneth cells, HD-5 has also been shown to be expressed in the female reproductive tract and in a rare intestinal epithelium cell type, similar to mucin producing goblet cells (Quayle *et al.*, 1998; Cunliffe *et al.*, 2001).

In contrast to α-defensins, β-defensins are more widely expressed mainly by the epithelial lining of the skin, kidney, respiratory tract, pancreas, reproductive tract, conjunctiva, urinary-, and GI tract (Table 1.2) (Klotman and Chang, 2006; Pazgier *et al.*, 2006). Expression by monocytes, macrophages and DCs has also been reported (Duits *et al.*, 2002). Although the human genome suggest the presence of more than 30 human β-defensin (hBDs), to date expression and regulation of only four human β-defensins (hBD-1 to -4) have been described (Schutte *et al.*, 2002; Ganz, 2003; Patil *et al.*, 2005; Pazgier *et al.*, 2006). The first human β-defensin hBD-1 was isolated from the serum of patients with renal disease (Bensch *et al.*, 1995). Subsequently, hBD-2 and hBD-3 were isolated from psoriatic scales with their structure and function now being well characterised (Harder *et al.*, 1997a; Garcia *et al.*, 2001b). Expression of hBD-4 has been demonstrated at the mRNA level only (Garcia *et al.*, 2001a). A synthetic form of

the peptide has been characterized further (Garcia *et al.*, 2001a), however, isolation of natural hBD-4 has not been reported.

| Distribution and source of defensins | | | |
|---|---|---|---|
| **Defensin** | **Tissue distribution** | **Cell source** | **Synthesis and regulation** |
| **HNP1-3** | Placenta, intestinal mucosa and cervical mucus plug | Neutrophils*, monocytes macrophages, natural killer cells, B cells and γδ T cells | Constitutive |
| **HNP4** | Not determined | Neutrophils* | Constitutive |
| **HD5 and HD6** | Salivary glands, small bowel, inflamed large bowel, stomach, eye, female genital tract (HD5 only), breast milk and inflamed urethral lumen | Intestinal Paneth cells* and vaginal epithelial cells (HD5 only) | Constitutive or inducible, such as by sexually transmitted infection |
| **HBD1** | Oral and nasal mucosa lungs, plasma, salivary glands, small and large bowel, stomach, skin, eyes, mammary glands, urogenital tract and kidneys | Epithelial cells*, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes | Constitutive or inducible in response to interferon-γ, lipopolysaccharide and peptidoglycan |
| **HBD2 and HBD3** | Oral and nasal mucosa, lungs, plasma, salivary glands, small and large bowel, stomach, skin, eyes, mammary glands, urogenital tract and kidneys | Epithelial cells*, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes | Inducible in response to viruses, bacteria, lipopolysaccharide, peptidoglycan, lipoproteins, cytokines (IL-1β, TNF) and growth factors |
| **HBD4** | Gastric antrum and testes | Epithelial cells* | Constitutive or inducible in response to PMA and bacteria |
| *Main cellular source. HBD, human β-defensin; HD, human α-defensin; HNP, human neutrophil peptide; IL-1β, interleukin-1β; PMA, phorbol 12-myristate 13-acetate; TNF, tumour-necrosis factor. | | | |

**Table 1.2: Tissue distribution, cell source and regulation of human α- and β-defensins (from Klotman and Chang, 2006).**

### 1.2.3 Transcriptional regulation

Our current understanding of the transcriptional regulation of epithelial α-defensins (HD-5 and -6) is limited mainly due to the lack of availability of a Paneth cell derived cell line. Under most conditions, expression of α-defensins is constitutive. However, transcriptional regulation seems to occur partly through factors intimately linked to cellular differentiation. Specifically, Tcf7-L2 has been identified as key transcription factor involved in Paneth cell α-defensin expression (Clevers, 2006). Activity of this transcription factor is liked to the Wnt/β-catenin signalling pathway, a central regulator for Paneth cell differentiation (van Es *et al.*, 2005). Furthermore, HD-5 and -6 have been found to be present prenatally in human Paneth cells. However, levels are low in human neonates and increase dramatically with maturation, suggesting that regulatory mechanism(s) inducing their expression are likely to be operative (Mallow *et al.*, 1996). In contrast to α-defensins, transcriptional regulation of β-defensin gene expression has been studied extensively. HBD-1 is expressed constitutively in various epithelial cells including the lining of the intestinal tract (O'Neil *et al.*, 1999). However, expression can be further modulated by infection and inflammation. In the GI tract constitutive hBD-1 gene and peptide expression has been shown to be down-regulated during infection with *Shigella* (Islam *et al.*, 2001) and *Cryptosporidium parvum* (Zaalouk *et al.*, 2004). In contrast, hBD-1 induction in inflamed *Helicobacter pylori* (*H. pylori*)-infected gastric mucosa has been reported (Bajaj-Elliott *et al.*, 2002). HBD-2 and hBD-3 are inducible in nature with induction occurring in response to bacterial infection or proinflammmatory stimuli. Stimulators for hBD-2 include IL-1β, TNF-α, INF-γ, phorbol 12-myristate 13-acetate (PMA), 1.25-dihydroxyvitamin D3 and bacterial LPSs (Table 1.3). In the GI epithelium hBD-2 has been shown to be induced by various enterophathogens such as *H. pylori*, *Salmonella enteritidis*, the probiotic bacterium *E. coli* Nissle 1917 and in states of chronic inflammation such as ulcerative colitis (UC)

(Wada *et al.*, 1999; Ogushi *et al.*, 2001; Bajaj-Elliott *et al.*, 2002; Wehkamp *et al.*, 2003b; Wehkamp *et al.*, 2004a) . Similar to hBD-2, expression of hBD-3 is induced by various inflammatory mediators (TNF-α, INF-γ) as well as microbes including bacteria and yeast (Table 1.2) (Dhople *et al.*, 2006). Expression of hBD-4 can be both constitutive and/or inducible, with induction following a similar pattern than hBD-2 and -3 (Table 1.2) (Pazgier *et al.*, 2006; Semple *et al.*, 2006).

### 1.2.4 Signalling pathways and host receptors involved in defensin expression

Variable expression and induction patterns of defensins suggest involvement of multiple signalling pathways in the regulation of these AMPs. Promoter analysis shows the presence of consensus NF-IL6 and interferon-γ (INF-γ) sites in hBD-1 promoter (Diamond *et al.*, 2000). Similarly, hBD-2 promoter contains multiple binding sites for NF-κB, activated protein (AP)-1, AP-2 and NF-IL6 (Yang *et al.*, 2004). Induction of hBD-2 expression in various tissues by cytokines and bacterial components has been shown to be mediated through activation of NF-κB, sometimes assisted by activated AP-1, MAP-kinases and by protein kinase C (PKC) (Krisanaprakornkit *et al.*, 2000; Mineshiba *et al.*, 2005; Wehkamp *et al.*, 2006). In contrast to hBD-2, hBD-3 does not contain any NF-κB binding sites in its promoter suggesting pathways distinct from those for hBD-2 expression might be responsible (Boughan *et al.*, 2006). Induction of hBD-3 has been shown in response to transforming growth factor alpha (TGF-α), EGF receptor transactivation and insulin-like growth factor 1 (IGF-1) (Sorensen *et al.*, 2003; Boughan *et al.*, 2006).

Several studies have demonstrated that TLRs mediate β-defensin expression. HBD-2 mRNA and peptide induction in airway epithelia and hBD-3 induction in keratinocytes occurs *via* TLR2 and its ligand lipopeptide (Hertz *et al.*, 2003; Wang *et al.*, 2003; Sumikawa *et al.*, 2006). In the GI tract both TLR2 and TLR4 have been implicated in

hBD-2 expression (Vora *et al.*, 2004). TLR3 agonists polyI:C and double-stranded RNA activate β-defensin expression in uterine and airway epithelia (Proud *et al.*, 2004; Schaefer *et al.*, 2005). In addition to TLRs, a second family of PRRs named nucleotide binding oligomerisation domain (NOD) like receptors, have been implicated in mediating human α- and β- defensin expression in the GI tract. Unlike TLRs, which are located either on the cell surface or in the cytoplasm, NODs are exclusively intracellularly located (Inohara *et al.*, 2005). NOD1 (encoded by the caspase-recruitment domain 4 gene; *CARD4*) and NOD2 (encoded by *CARD15*) recognize the intracellular presence of peptidoglycan (the major component of the cell wall of Gram-positive and Gram-negative bacteria (Inohara and Nunez, 2003; Inohara *et al.*, 2005; Franchi *et al.*, 2006). *H. pylori* has been shown to induce IL-8 and hBD-2 expression *via* activation of NOD1 (Viala *et al.*, 2004; Boughan *et al.*, 2006). Voss and colleagues identified NOD2 as a major intracellular PRR for hBD-2 in human embryonic kidney cell-line and primary keratinocytes. Induction of hBD-2 required functional binding sites for NFκ-B and AP1 in the hBD-2 promoter (Voss *et al.*, 2006). A role for NOD1 as a major sensor for enteroinvasive *Escherichia coli* (*E. coli*) was proposed by Kim and colleagues (Kim *et al.*, 2004), while NOD2 has been implicated in IEC defence against *Salmonella* (Hisamatsu *et al.*, 2003b), further highlighting the role of these PRRs in microbial sensing and mediating innate host defence. Although mechanism(s) by which HD5 and HD6 expression is modulated in response to bacteria remains unclear, several studies suggest NOD2 is required for peptide expression *in vivo* (Lala *et al.*, 2003; Wehkamp *et al.*, 2004b).

### 1.2.5 Peptide processing and secretion

Both α- and β-defensins are translated as large precursor polypeptide chains containing a signal peptide, a propiece and the mature peptide. Following transport to the Golgi

body, the hydrophobic signal peptide is proteolytically cleaved. Cleavage of the propiece varies between defensin families. The signal peptide of HNP1-4 is proteolytically removed together with the propiece in the Golgi body, followed by storage of the mature peptide in neutrophil granules (Ganz *et al.*, 1985). In contrast, HD5 and HD6 are stored in specialized granules in an inactive precursor form (propiece and mature peptide), in close proximity to the inactive form of the processing enzyme, trypsin (trypsinogen). On appropriate stimuli (bacterial products or cholinergic agonists) (Ayabe *et al.*, 2000; Ouellette, 2006), the inactive defensin proform is secreted into the intestinal crypt where trypsin mediates the enzymatic removal of the propiece and activates the precursor protein (Ayabe *et al.*, 2000; Ghosh *et al.*, 2002). Serine protease inhibitors (also produced and released by Paneth cells) add further complexity to the regulation of HD-5 and HD-6 by controlling the enzymatic activity of trypsin in the lumen of the intestinal crypt (Ghosh *et al.*, 2002).

At present enzyme(s) involved in post-translational modifications of β-defensins are unknown. Although β-defensins are secreted upon induction, recent evidence suggest that intracellular storage and activity may also occur (Sorensen *et al.*, 2005).

### 1.2.6 Function and effector mechanisms

#### 1.2.6.1 Antimicrobial activity and mode of action

Defensins exhibit broad-spectrum antimicrobial activity against bacteria, fungi, protozoa and enveloped viruses reflecting their role in innate defence. Defensins in general are salt sensitive, exhibiting maximum bactericidal activity in buffers of low ionic strength and neutral pH. *In vivo* physiological salt concentrations might act as natural regulators of their antimicrobial action restricting their activity to sequestered environments such as the immediate epithelial surface of skin and GI tract. Although the exact concentration of defensins at their expression site is not known, *in vitro*

studies suggest effective microbicidal activity at concentrations in the range of 0.5-5 μM (Selsted and Ouellette, 2005).

Our understanding of the mechanism(s) by which defensins mediate their antimicrobial activity is still incomplete. It is generally accepted that bactericidal activities of AMPs depends upon their interaction with the microbial cell membrane (Hancock and Rozek, 2002). The first step in this interaction is the attraction between the peptide and the target microbe involving electrostatic and/or hydrophobic bonding. The cationic and amphiphatic nature of defensins plays a key role in selectively interacting with hydrophilic, negatively charged microbial membranes as opposed to eukaryotic cells, in which uncharged lipids predominate (Jenssen *et al.*, 2006). Events that occur following initial AMP membrane interaction are less well defined and several models have been put forward including carpet hole, barrel-stave, toroidal pore and the aggregated model (Figure 1.5). In each model system the peptide either causes target cell depolarization followed permeabilization, and/or translocates across the membrane into the cytoplasm. While the first mechanism most likely leads to irreversible damage of the target membrane potentially followed by leakage of cytoplasmic contents, peptide translocation across the membrane can have several implications. A growing number of peptides have been shown to translocate across the membrane and accumulate intracellularly, where they target a variety of essential cellular processes to mediate cell killing including inhibition of nucleic acid-, protein-, cell wall synthesis and induction of autolytic enzymes (Figure 1.5) (Sahl *et al.*, 2005; Jenssen *et al.*, 2006).

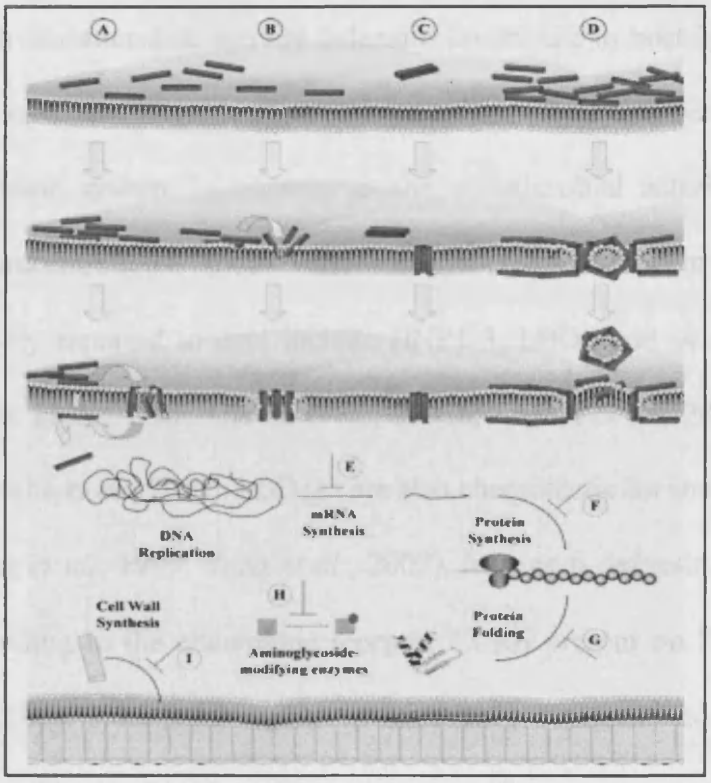



**Figure 1.5: Mechanisms of action of antimicrobial peptides.** Several models of membrane permeablilization are demonstrated (A-D). Mechanisms of action independent of bacterial membrane permeabilization are summarized in E to I (from Jenssen *et al.*, 2006).

A: Aggregate model;

B: Toroidal pore model;

C: Barrel-stave model;

D: Carpet model;

E: Inhibition of DNA/RNA synthesis;

F: Interference with protein synthesis;

G: Interference with protein folding;

H: Inhibition of enzymes involved in aminoglycosides modification;

I: Inhibition of cell wall synthesis.

1.2.6.2 Additional functions

In addition to their antimicrobial activity defensins contribute to host innate defence in multiple ways. Their chemotactic properties form an important link between the innate and adaptive immune system. In contrast to the antimicrobial activity of defensins requiring μM concentrations, the chemotactic activity occurs in a nM range. Chemotactic activity reported to date include HNP1-3, hBD3 and -4 chemoattracting monocytes (Territo *et al.*, 1989; Garcia *et al.*, 2001b; Garcia *et al.*, 2001a) and hBD2 mast cells (Niyonsaba *et al.*, 2002). hBD1-3 are also chemotactic for immature dendritic cells (iDCs) (Yang *et al.*, 1999; Yang *et al.*, 2002). Murine β-defensin 2 mediates this interaction *via* binding to the chemokine receptor CCR6 present on DCs and T cells (Biragyn *et al.*, 2001). Additional studies in mice have demonstrated that α- and β-defensins are very effective in promoting antigen specific immune responses (Brogden *et al.*, 2003; Yang *et al.*, 2004). Further functions of defensins include degranulation of mast cells, induction of neovasculogenesis and enhanced wound closure (Figure 1.6) (Selsted and Ouellette, 2005).

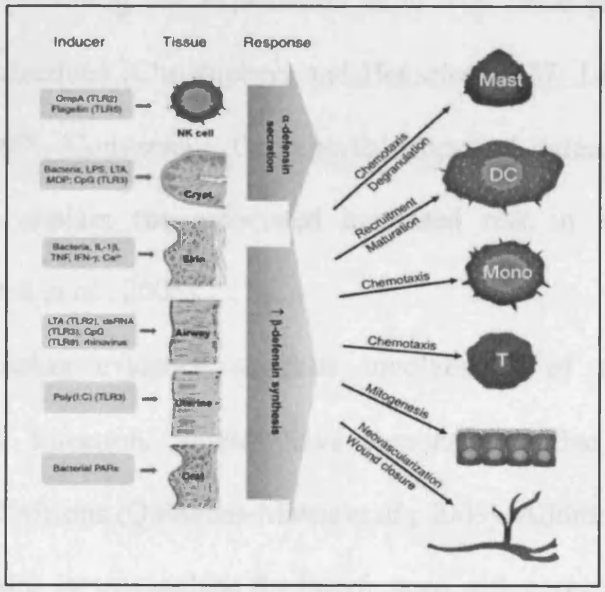



**Figure 1.6: Mobilization, induction and interactions of defensins (from Selsted and Ouellette, 2005).**

### 1.2.7 Role in health and disease

The pathogenesis of several infectious diseases and associated medical conditions have been linked to expression and function of defensins. Amongst the first to be reported was cystic fibrosis. Due to a genetic defect in a chloride channel, luminal salt concentrations in the airway surface fluid are increased, inhibiting the antimicrobial activity of hBD1 (Smith *et al.*, 1996; Goldman *et al.*, 1997). This local impairment of host defence is thought to contributes to the increased susceptibility of these patients to chronic bacterial infections (Goldman *et al.*, 1997). Additionally, increased defensin levels have been observed in inflammatory lung diseases, such as diffuse panbroncheolitis (DPB), idiopathic pulmonary fibrosis (IPF) and acute respiratory distress syndrome (ARDS), further highlighting a potential involvement of defensins in disease pathogenesis (Aarbiou *et al.*, 2002).

More insight into the role of defenins *in-vivo* has been provided by investigating defensin expression in human skin. HBD-2 and LL-37 (the only human cathelicidin) have been shown to be expressed in high levels in keratinocytes of patients suffering from psoriasis, providing one explanation as to why these patients are less prone to bacterial skin infections (Christophers and Henseler, 1987; Liu *et al.*, 1998; Schroder and Harder, 1999). Conversely, the reported impaired defensin expression in atopic dermatitis may explain the associated increased risk in acquiring bacterial skin infections (Howell *et al.*, 2005).

Recently, increasing evidence suggests involvement of α- and β-defensins in controlling HIV infection. Studies have demonstrated that defensins can directly inactivate HIV-1 virions (Quinones-Mateu *et al.*, 2003). Additionally, reported anti HIV effects of defensins *in-vivo* include the interference with coreceptor(s) required for viral entry, ultimately leading to reduced replication (Munk *et al.*, 2003). Besides HIV,

defensins exhibit antiviral activity against other viruses further highlighting their critical role in host protection against virally mediated disease (Klotman and Chang, 2006).

Increasing evidence suggests that genetic factors such as polymorphisms and/or number of defensin gene copies influence defensin expression and possibly function. The gene copy number of α- and β-defensins varies amongst individuals with increased copy numbers correlating with high peptide expression *in vivo* (Linzmeier and Ganz, 2005), suggesting genetic variation may contribute to host susceptibility to infection and disease. Single nucleotide polymorphisms (SNPs) are a recognized feature of hBD-1 gene (*DEFB1*) and recent reports have linked their presence to the susceptibility of developing atopic dermatitis, chronic obstructive pulmonary disease (COPD) and asthma (Matsushita *et al.*, 2002; Levy *et al.*, 2005; Prado-Montes de Oca *et al.*, 2007). These findings are supported by *in vivo* studies demonstrating that deletion of murine β-defensin-1 gene (*Defb1*) results in defective clearance of *Haemophilus influenzae* from the lung (Moser *et al.*, 2002). Furthermore, a study by Morrison and colleagues showed increase in *Staphylococcae* species in the bladders of *Defb1* K/O mice (Morrison *et al.*, 2002).

Due to the constant exposure of the intestinal epithelium to a wide range of commensal bacteria, defensin expression at this mucosal surface is likely to not only effect host susceptibility to enteropathogens but also influence bacterial flora. Increasing evidence suggest that differential expression of defensins in the small and large bowel may have a significant impact on the content and type of resident bacterial flora (Salzman *et al.*, 2007). Furthermore, high expression levels of enteric α-defensins in the lumen of small intestinal crypts provide sterility, ensuring protection of stem cells found at the bottom of the intestinal crypt (Bevins *et al.*, 1999).

Studies in mice and humans have highlighted a role for defensins in GI innate defence against infections. Cryptidin (mouse alpha-defensin) maturation was abrogated in the

small intestine by targeting the matrilysin gene (*MMP7*, enzyme responsible for processing murine pro-alpha-defensin). *MMP7* null ($Mmp^{7-/-}$) mice lacked detectable mature cryptidins which led to increased susceptibility to oral challenges with enteric bacteria including *E. coli* and *Salmonella typhimurium* (*S. typhimurium*) (Wilson *et al.*, 1999). Conversely, a transgenic mouse expressing HD-5 was found to be immune to the oral challenge with virulent *S. typhimurium* (Salzman *et al.*, 2003). In humans increased risk of infectious diarrhoea in an African cohort with decreased α-defensin expression has been noted (Kelly *et al.*, 2006).

Both enteric α- and β-defensins have been implicated in disease pathogenesis of chronic inflammatory bowel diseases (IBD), i.e. ulcerative colitis (UC) and Crohn's disease (CD). While UC is generally restricted to the colon, CD frequently affects the ileum of the small bowel and the colon. Studies initiated by Wehkamp and colleagues have demonstrated decreased expression of α-defensins HD5 and HD6 in patients suffering from ileal CD (Wehkamp *et al.*, 2005). Independent studies have shown expression of the intracellular PRR NOD2 in Paneth cells and established a link to α-defensins (Lala *et al.*, 2003). Importantly, about one third of CD patients are known to have a mutation in the gene coding for NOD2, and particularly those patients were found to express low levels of HD5 and HD6 (Wehkamp *et al.*, 2004b).

In addition to Paneth cell defensins, expression of human β-defensins has also been found to be impaired in CD patients. Specifically, induction of hBD-3 and hBD-4 was found to be significantly reduced in the inflamed colonic mucosa of CD patients when compared to samples taken from individuals suffering from UC (Wehkamp *et al.*, 2003b). More recently, Fellermann and colleagues were able to demonstrate a correlation between low hBD-2 copy numbers and peptide expression *in vivo*, leading to a significantly increased risk for the development of CD (Fellermann *et al.*, 2006).

Taken together these findings highlight the important role of defensins during GI infection and inflammation, significantly contributing to disease susceptibility and outcome.

# AIMS & HYPOTHESIS

The main aim of this thesis is to further our understanding of the role and regulation of intestinal host innate immunity during *C. jejuni* infection. We hypothesise that modulation of intestinal innate host defence by *C. jejuni* contributes to the self-limiting nature of disease. Identification of molecular mechanism(s) leading to clearance of the infective agent may help us identify susceptible individuals and develop new preventive and/or therapeutic strategies in the future.

## The specific aims of this project are:

1) **To delineate the modulation of intestinal human β-defensin expression during *C. jejuni* infection.**

2) **To test susceptibility of *C. jejuni* to human β-defensins.**

3) **To investigate the potential role of *C. jejuni* capsular polysaccharide in modulating innate host defence and providing protection against β-defensins.**

4) **To identify the potential role of the intracellular PRRs NOD1 and NOD2 in mediating intestinal innate host response to *C. jejuni.***

5) **To develop an *ex-vivo* infection model and improve existing *in-vitro* models to investigate *C. jejuni* disease pathogenesis.**

# CHAPTER 2

# Materials and Methods

## 2.1 Mammalian cell culture

### 2.1.1 Cell culture reagents

All plastic including 6, 12, 24, 96 well plates, 25 $cm^2$, 75 $cm^2$ tissue culture flasks, pipettes and polypropylene coated Eppendorf tubes were purchased from Fisher Scientific, Leicestershire, UK. Cell culture reagents were purchased from Invitrogen, Paisley, UK, unless otherwise stated.

### 2.1.2 Epithelial cell lines

The human intestinal epithelium cell (IEC) lines Caco-2, HT-29 and the larynx carcinoma cells Hep-2 were used throughout the study. Caco-2 and HT-29 cells are derived from colorectal adenocarcinoma and upon reaching confluence, express characteristics of enterocytic differentiation. Both IEC lines were maintained in DMEM +glutamax culture medium, supplemented with 10% heat inactivated fetal calf serum (FCS; Sigma, Poole, UK), 100 units/ml penicillin, 100μg/ml streptomycin, and 1% non-essential amino acids. This media is referred to as complete media thereafter. Similarly for Hep-2 cells, Minimal Essential Media (MEM) was supplemented with 2mM L-glutamine plus above. The cells were maintained at 37°C in a 5% $CO_2$ humidified incubator (Galaxy $CO_2$ Incubator, Wolf Laboratories Ltd., Pocklington, UK).

### 2.1.3 Cell passage

Cells were examined regularly under the microscope and checked for morphological features of growth as well as contamination. Cells were passaged upon reaching 80-90% confluency. Cells were washed twice with sterile phosphate buffered saline (PBS) before addition of Trypsin-EDTA for 5-10min at 37°C. This treatment allowed detachment of adherent cells. Trypsin activity was inactivated by the addition of complete media containing 10% FCS. Resultant cell suspension was centrifuged at

1200rpm for 10min at 4°C (Rotina 46R centrifuge, Wolf Laboratories, UK). The cell pellet was resuspended in complete media and re-centrifuged in order to remove any remaining trypsin. Finally, cells were seeded at an appropriate cell density and maintained in $25cm^2$ or $75cm^2$ culture flasks, 6 or 96 well plates as required.

### 2.1.4 Counting viable cells

Cells were quantified as follows: 10µl of resuspended cells were mixed with an equal volume of 0.4% trypan blue (Sigma, Poole, UK). 10µl of this mixture was placed onto a haemocytometer (Hawksley+Sons Limited, West Sussex, UK) counting chamber, and viable cells counted (Zeiss Microscope, Germany) at a magnification of x 40. Non-viable cells were distinguished from viable cells by the uptake of dye. Concentration of viable cells was determined as:

Number of cells
within 25 squares x dilution factor (2) = Total number of cells x $10^4$ cells/ml

### 2.1.5 Freezing cells

For long-term storage, confluent adherent cell lines were trypsinised, centrifuged and resuspended in media containing 80% FCS, 10% complete media and 10% sterile dimethyl sulphoxide (DMSO; Sigma, Poole, UK) and transferred into cryovials (Nunc, Roskilde, Denmark). To maintain greater viability and integrity cells were cooled gradually over a 24h period prior to long-term storage at -80°C or liquid nitrogen.

### 2.1.6 Thawing cells from -80°C storage

Cryovials were removed from -80°C storage and placed in a 37°C water bath to achieve rapid thawing. Complete media (37°C) was added and cells centrifuged (1200rpm, 10min, 4°C) to remove DMSO. The cell pellet was resuspended in complete media and

transferred into a 25cm$^2$ tissue culture flask. After overnight incubation, non-viable cells were removed by change of media and adherent cells were allowed to grow.

## 2.2 Bacterial culture

### 2.2.1 Preparation of blood agar plates

Bacterial reagents for maintaining *C. jejuni* strains including horse blood, agar and selective supplements were purchased from Oxoid, Basingstoke, UK. Wild type (WT) *C. jejuni* strains were grown on blood agar plates prepared using Columbia Agar Base (39g/L), supplemented with 5% horse blood and *C. jejuni* selective (Dent) supplement. *C. jejuni* selective supplements contained Vancomycin (10mg/L), Polymyxin B (2,500 IU/L), Trimethoprim (5mg/L), Amphotericin B (2mg/L), Cephalothin (15mg/L). One vial of supplement was resusupended by adding 2ml of aseptic, sterile PBS and mixed gently to dissolve. The contents were then added to 500ml of Columbia Blood Agar Base cooled to 50-55°C and mixed well before pouring into sterile Petri dishes (Fisher Scientific, Leicestershire, UK).

### 2.2.2 *C. jejuni* strains

All *C. jejuni* strains used throughout this study were provided by Dr. N. Dorrell and Mr. A. Elmi (LSHTM) and are listed in Table 2.1. *C. jejuni* strains were grown at 37°C in a microaerobic chamber (Don Whitley Scientific Ltd, Shipley, UK) containing 85% $N_2$, 5% $O_2$ and 5% $CO_2$ on blood agar plates or in Mueller-Hinton (MH) broth. A sterile loop was used to streak bacteria onto blood agar plates.

Prior to experiments, *C. jejuni* was routinely grown on blood agar plates over 24h, providing a "mixed" culture bacterial population with organisms in various phases of growth. Bacteria referred to as "mid-log" phase were transferred from 24h blood agar

plates into MH broth and grown over an additional 16h leading to a bacterial population mainly consisting of mid-log phase organisms.

| ***C. jejuni* strain** | **Characterisation** | **Reference** |
|---|---|---|
| WT 11168H | Hypermotile clinical isolate. Derived from the sequenced strain *C. jejuni* NCTC 11168. Shows high levels of intestinal colonization in a chicken colonization model. | (Jones *et al.*, 2004) |
| *kpsM* | Isogenic capsule-deficient mutant of 11168H. Deficient in *kpsM*. | (Karlyshev *et al.*, 2000) |
| WT 81-176 | Clinical isolate from a multistate gastroenteritis outbreak | (Korlath *et al.*, 1985) (Black *et al.*, 1988) |

**Table 2.1: *Campylobacter jejuni* strains, clinical isolates and isogenic mutant utilised in the present study.**

### 2.2.3 Quantification and inactivation of *C. jejuni*

Bacterial colonies were resuspended in antibiotic free media supplemented with 1% FCS (infection media) using a sterile bacterial spreader. Suspension was diluted 10-fold and optical density (OD) measured spectrophotometrically at 600nm (6300 Spectrophotometer, Jenway, UK). According to initially performed viable counts, a suspension with an $OD_{600}$ of 1 contained approximately $1x10^9$ colony forming units (CFU) per ml. Bacterial inactivation was achieved by fixation in 4% paraformaldehyde (PFA) in PBS for 15min followed by 3 washes in infection media. 100% inactivation was confirmed by viable counting.

## 2.3 Bacterial and cytokine stimulation of Intestinal Epithelium Cells (IECs)

For bacterial co-culture experiments cells were set up in 6 well plates or $25cm^2$ tissue culture flasks and grown to 80-90% confluency. 16-24h prior to exposure to bacteria, cells were serum starved by the replacement of complete media with infection media. Infections were routinely conducted at a multiplicity of infection (MOI) of 100 [i.e., $1x10^6$ epithelial cells:$1x10^8$ bacteria] unless stated otherwise. MOI of 100 was chosen as previous studies have shown maximum expression of epithelial host defence genes under these conditions (Bajaj-Elliott *et al.*, 2002).

Cytokine stimulation experiments were undertaken in media supplemented with 1% FCS, in the presence of antibiotics. Recombinant cytokines (Peprotech, London, UK) were reconstituted according to manufacturer's instructions and used at the following concentrations: Interleukin-1β (IL-1β) and tumour necrosis factor- α (TNF-α): 20ng/ml, interferon-γ (IFN-γ): 40ng/ml (~100U/ml). At the end of the experimental time-period, media was removed, followed by two washes with sterile PBS. Cells were either stored at -80°C until required or used immediately for RNA extraction.

## 2.4 RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR)

All reagents for RNA extraction, reverse transcription and polymerase chain reaction (PCR) were obtained from Invitrogen, Paisley, UK unless stated otherwise.

### 2.4.1 Total RNA extraction and quantification

For total cellular RNA isolation a monophasic solution of phenol and guanidine thiocyanate (TRIZOL) was utilized, of which 1ml was added directly to cell monolayers. Detachment and homogenization of cells was achieved by gentle shaking (Rotatest, Denley, UK) for 10min at RT. The viscous solution (due to cellular DNA) was transferred into a sterile 1.5ml eppendorf tube. Vigorous repeated dispersion of the solution through a syringe and needle (25gauge, Terumo, Leuven, Belgium) allowed further homogenization of cellular contents. The resultant Trizol solution was allowed to stand at RT for 5min to permit greater dissociation between nucleic acid-protein complexes. 200µl chloroform (Sigma, Poole, UK) was added to each 1ml Trizol and mixed thoroughly (Vortex Genie-2, Scientific Industries, NY, USA) for 30sec before centrifugation (13000rpm, 20min, 4°C; Eppendorf 5415R microfuge, Eppendorf AG, Hamburg, Germany). This organic extraction procedure allows total RNA to remain in the aqueous phase leaving denatured proteins at the interface and DNA in the organic phase. Following centrifugation, the top aqueous layer was aspirated (approximately 400µl) and transferred into a fresh eppendorf tube. RNA was precipitated by the addition of an equal volume of isopropanol (Sigma, Poole, UK) and stored at -20°C overnight. Total RNA was pelleted by centrifugation (13000rpm, 20min, 4°C) and washed with ice-cold 70% ethanol (BDH Laboratories, Poole, UK). The RNA pellet was either vacuum (Heto Vacuum Centrifuge, Jencons PLC, UK) or air-dried to remove traces of ethanol prior to resuspension of RNA in RNase- free water (Sigma, Poole,

UK). Resuspended RNA was quantified by spectrophotometry. RNA concentration was determined by absorbance (U-1800 Spectrophotometer, Digilab Hitachi, Tokyo, Japan) measured at 260nm and 280nm. The amount of RNA (μg/μl) was calculated as follows: **[(reading at 260nm) x (dilution factor) x 40] / 1000**.

The RNA ratio at 260/280 also allowed the assessment of the degree of purity of the RNA preparation. Pure RNA gives a value of 1.8 at 260/280 nm (Sambrook *et al.*, 1989). Integrity of extracted RNA was also confirmed by gel electrophoresis. 5μg of each sample was routinely run on 1% agarose gels (Invitrogen, Life Technologies, Paisley, UK) in Tris Borate EDTA (TBE) buffer (Sigma, Poole, UK). Bands were visualized by ethidium bromide (Sigma, Poole, UK) staining. Sharp, clear 28S and 18S rRNA bands were indicative of good quality total RNA. A representative RNA gel is shown in Figure 2.1.

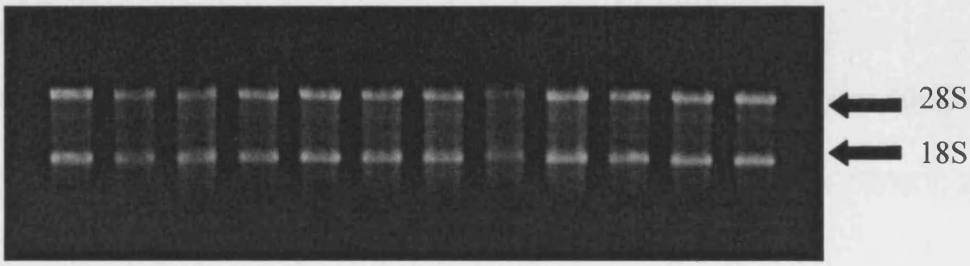



**Figure 2.1: Total cellular RNA from IEC Caco-2 cell line.** A representative series of RNA preparations is shown. 28S and 18S rRNA subunits are clearly defined with minimal evidence of RNA degradation.

### 2.4.2 Reverse transcription

5μg of total cellular RNA was reverse transcribed to complementary DNA (cDNA) using Moloney murine leukaemia virus reverse transcriptase enzyme. In a typical reverse transcription reaction of 20μl total volume, 1μl (0.5μg) oligo-dT was added to 5μg of RNA and heated to 70°C for 10min followed by immediate chilling on ice to enhance specific annealing between oligo-dT primer and the poly-A tail of mRNA molecules.

The remaining components of the reaction mix:

| | |
|---|---|
| 5 x First strand buffer | 4μl |
| 0.1M DTT | 2μl |
| deoxynucleoside Triphosphates (dNTPs) | 1μl |
| 100U Enzyme (reverse transcriptase) | 1μl |

were added to the RNA : oligo-dT complex and the reaction allowed to proceed at 42°C for 1h. Reaction was terminated by heat inactivation at 70°C for 10min.

### 2.4.3 Polymerase chain reaction (PCR)

cDNA obtained was used in subsequent PCR reactions. Each reaction volume was 50μl, comprising the following components:

| | |
|---|---|
| 10 x PCR Buffer [200mMTris-HCl (pH8.4), 500mM KCL] | 5μl |
| 50mM $MgCl_2$ | 1.5μl |
| 10mM dNTP mix | 1μl |
| 20 pmol each oligonucleotide primer | 2μl |
| 0.5 units *Taq* polymerase | 0.2μl |
| cDNA | 1-5μl |

The remainder consisted of RNase free water to make up a total volume of 50µl. Master mixes were prepared for multiple reactions to minimize inter-sample variation. Sequences of innate defence gene primers utilised in this study are listed in Table 2.2. The contents of the PCR reaction were briefly centrifuged prior to amplification in a thermal cycler (Peltier Thermal Cycler, MJ Research, Massachusetts, USA). Conditions were:

a) 94°C for 3min to denature the template - 1 cycle

b) 94°C denaturation for 90sec
   60°C annealing for 90sec
   72°C extension for 90sec
   } - 32-37 cycles

c) 72°C for 10min - 1 cycle

PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Semi-quantitative analyses were performed by densitometric measurements of innate gene expression bands and normalized to the house-keeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Alpha Imager, Multimage Light Cabinet, Alpha Innotech Corporation, Essex, UK).

| Target gene | Sense primer | Anti-sense primer | Product size in (bp) |
|---|---|---|---|
| hBD-1 | [109]TTG TCT GAG ATG GCC TCA GGT GGT AAC[136] | [362]ATA CTT CAA AAG CAA TTT TCC TTT AT[337] | 253 |
| hBD-2 | [11]CCA GCC ATC AGC CAT GAG GGT CTT[34] | [287]CAT GTC GCA CGT CTC TGA TGA GGG AGC[261] | 276 |
| hBD-3 | [202]GGT GAA GCC TAG CAG CTA TGA GGA TC[227] | [385]GAG CAC TTG CCG ATC TGT TCC TCC[362] | 183 |
| Lysozyme | [108]GAA CTC TGA AAA GAT TGG GAA TGG A [132] | [463]ACA ACC TTG AAC ATA CTA ACG GAC A[439] | 355 |
| IL-8 | [41]ATG ACT TCC AAG CTG GCC GTG[62] | [308]TCT CAG CCC TCT TCA AAA ACT TCT C[332] | 291 |
| NOD1 | [2209]GTT GTC CAA AGC CAA ACA GAA ACTC [2233] | [2371]GCC CAC GTT CAT CTG GAT GCTG[2392] | 183 |
| NOD2 | [187]ATG TGC TCG CAG GAG GCT TTT CAG GCA [213] | [988] GTG CTG GTG GTG GGT GAG GCG [1008] | 823 |
| GAPDH | [630]CTA CTG GCG CTG GCA AGG CTG T[651] | [989]GCC ATG AGG TCC ACC ACC CTG CTG[966] | 359 |

**Table 2.2: Sequences of synthetic oligonucleotide primers utilized in this study.**

## 2.5 Protein analysis

Equipment and reagents for Western blotting were from Bio-Rad Laboratories, Hemel Hempstead, UK and Amersham Biosciences, St Albans, UK. A detailed list of all gel components and buffer recipes used is provided in Table 2.3 and 2.4 respectively. Antibodies used are listed in Table 2.5.

| **Gel Components** | **16% Resolving gel** Component Volume (~30ml ) 1 large gel | **5% Stacking gel** Component Volume (~20ml) for 2 gels |
|---|---|---|
| Water | 5.9 | 10.0 |
| Glycerol | 4g (≅ 3.17ml) | None |
| 30% Acrylamide mix (Protogel, National Diagnostics, UK) | 10.8 | 3.4 |
| 3 M Tris-Cl (pH 8.45) | 10.0 | 6.2 |
| 10% SDS | 0.3 | 0.2 |
| 10% Ammonium persulphate | 0.05 | 0.05 |
| TEMED | 0.015 | 0.025 |

**Table 2.3: Composition of Tris-Tricine SDS- Polyacrylamide Gels.**

| Buffer | Composition |
|---|---|
| **SDS Sample buffer** | (1x) 200mM Tris-HCl (pH 6.8), 40% glycerol, 2% SDS, 0.04% Coomassie Blue |
| **Electrophoresis Buffer (pH 8.3)** | 100mM Tris, 100mM Tricine, 0.1% SDS |
| **Transfer Buffer** | 100mM Tris, 100mM Tricine |
| **Blocking buffer** | 1 x TBS/0.1 % Tween-20, 5% w/v nonfat dry milk (Marvel) |

**Table 2.4 Buffer composition for Tris-Tricine SDS PAGE.** All reagents were purchased from Sigma or BDH Laboratories, Poole, UK.

| Primary Antibody | Source | Dilution | Company |
|---|---|---|---|
| hBD2 | Goat polyclonal IgG | 1:500 | Autogen Bioclear, Wiltshire, UK. |
| hBD3 | Rabbit polyclonal IgG | 1:1000 | Gentaur Molecular, Brussels, Belgium. |
| **Secondary Antibody** | | | |
| Anti-rabbit antibody conjugated to horseradish peroxidase | Goat anti-rabbit IgG | 1:2000 | Cell Signalling Technology, Boston, USA. |
| Anti-goat antibody conjugated to horseradish peroxidase | Rabbit anti-goat IgG | 1:2000 | Dakocytomation Ltd, Cambridgeshire, UK. |

**Table 2.5: Antibodies utilised in the present study.**

### 2.5.1 Intestinal epithelial β-defensin peptide extraction

Experiments were routinely set up in $25cm^2$ or $75cm^2$ tissue culture flasks and bacterial co-culture performed over 24h. Protein was extracted from both cells and supernatants of infected cells as well as uninfected controls. Supernatants were removed and glacial acetic acid (100%) added in appropriate amounts to achieve a final concentration of 20%. 4ml of 20% acetic acid was added to each $75cm^2$ tissue culture flask. Following overnight incubation on a rotary shaker (Rotatest shaker, Denley, UK), cell lysates and supernatants were transferred into 1.5ml eppendorfs, and homogenised by shearing and sonication (Bandelin Sonopuls, Bandelin Electronics, Berlin, Germany) for 10-15sec on a power setting of ~60%. Resuspended proteins post centrifugation (1.200g, 20min) were further concentrated by lypholization (Heto DryWinner, Allerod, Denmark). Lypholised protein contents were resuspended in 10nM acetic acid and quantified by Bradford assay as described below (Sigma, Poole, UK).

### 2.5.2 Protein quantification (Bradford assay)

Protein amounts were determined using a Bradford assay. Bovine Serum Albumin (BSA) standards of varying concentrations (2μg to 25μg) and protein samples were prepared in a volume of 1ml. Equal amount of Bradford reagent (Sigma, Poole, UK) was added to each sample, vortexed gently and left at 37°C for 30min, prior to OD measurements at 595nm. Protein concentration was determined using the standard curve plotted for BSA.

### 2.5.3 Tris-Tricine Sodium Dodecyl Sulphate Page Electrophoresis (SDS-PAGE)

Samples were prepared by the addition of tris-tricine loading buffer (Bio-Rad Laboratories, Hemel Hampstead, UK) and 10mM β-Mercaptoethanol prior to heating at 95-100°C for 5min. After cooling on ice and centrifugation, samples were subjected to

Tris-Tricine SDS-PAGE. Commercial Tris-tricine markers (Bio-Rad Laboratories, Hemel Hempstead, UK) allowed for molecular weight determination and a visual check for transfer efficiency. Recombinant peptides (hBD-2, 4.3kDa and hBD-3, 5.1kDa; Peprotech, Ltd., London, UK) served as positive controls. Typically, 20μg total protein was loaded and run at 100V for 2-4h. Protein detection was followed by silver staining (Bio-Rad Laboratories, Hemel Hampstead, UK). Following electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane at 0.8mA/cm$^2$ for 40min using a semi-dry transfer system. Prior to transfer, PVDF-membrane was prepared by soaking in 100% methanol followed by three washes in PBS. Membrane and gels were allowed to equilibrate in chilled transfer buffer for at least 5min. After transfer, UV cross-linking (auto-crosslink setting) was performed on membrane followed by blocking of non-specific binding in 5% non-fat milk/Tris-buffered saline (TBS, Sigma, Poole, UK) for 1-2h at RT with gentle shaking. This was followed by overnight incubation in anti-hBD-2 or hBD-3 antibody in blocking buffer on a suspension mixer at 4°C. After 3-4 washes in TBS with 0.1% Tween-20 (TBS/T) for 10min each, the blot was incubated in relevant secondary antibody for 1h on the rotary shaker at RT. Blots were washed 3-4 times for 10min with TBS/T before being exposed to an enhanced chemiluminescence (ECL) reaction for 5min, where 1ml of reagent A was added to 25μl of reagent B (ECL plus, Amersham Biosciences, UK). Excess solution was drained, the membrane placed between a sheet of clingfilm and exposed to an x-ray film (Hyperfilm ECL). Development of film ranged between 3-5min as necessary.

### 2.5.4 IL-8 Enzyme linked immuno-sorbent assay (ELISA)

IL-8 protein concentration was measured in cell supernatants using Quantikine Human CXCL8/IL-8 Immunoassay (R&D Systems, Abingdon, UK). All solutions, buffers and a 96 well polystyrene microplate coated with mouse monoclonal antibody against IL-8 were included in the kit. Analysis was performed according to instructions provided. Briefly, IL-8 standard was diluted in Calibrator Diluent to concentrations ranging from 2000 to 15.6pg/ml. 100µl of assay diluent (RD1-85) was placed in each well and 50µl of standard, control or sample added. Following 2h incubation at RT, wells were washed 4 times using 400µl of wash buffer. Complete removal of all liquid was ensured and wells incubated with 100µl of conjugate for 1h at RT. Following washes, 200µl of substrate solution was added and left for 30min prior to addition of 50µl stop solution. Optical density was measured within 30min using a microplate reader set at 450nm (MRX Revelation, MTX Lab System Inc., Virginia, USA) and amount of protein calculated according to standard curve.

## 2.6 Luciferase promoter-constructs and over-expression plasmids

*Renilla,* IL-8, and NF-κB promoter plasmids and pcDNA were generous gifts from Dr .Andrew Bowie (Trinity College, Dublin, Ireland). Luciferase reporter constructs of hBD-2 and hBD-3 were kind gifts from Mr Shao-ren Wang (Institute of Child Health, London, UK) and Dr Ole Sorensen (Lund University, Lund, Sweden) respectively. NOD2 overexpression plasmids were kindly provided by Professor G. Núňez (Department of Pathology and Comprehensive Cancer Centre, University of Michigan Medical School, Ann Arbor, Michigan, USA).

### 2.6.1 Transformation of plasmid DNA

Plasmid DNA was transformed into JM109 competent cells (Promega, Southampton, UK) according to the protocol provided. Plasmid DNA (1-10ng) was added to 50µl of thawed competent cells and left on ice for 30min. Sporadic flicking of the tube ensured DNA adherence to cell membranes. Cells/DNA complex were heat-shocked for 2min at 42°C and immediately placed on ice for a further 5min. 1ml LB (Luria Bertani) broth (Merck, Hertfordshire, UK) containing 10mM $MgCl_2$ and 10mM D-glucose was added and the mixture further incubated for 1h at 37°C with gentle shaking. Dilutions of the transformation reaction were plated (100µg/ml ampicillin in LB agar) and incubated overnight at 37°C. The following day colonies were analysed appropriately.

### 2.6.2 Purification of plasmid DNA

Plasmid DNA was purified using Qiagen (Crawley, West Sussex, UK) EndoFree Plasmid Maxi Kit, which included instructions and all reagents. Briefly, a single colony was used to inoculate a starter culture of 5ml LB broth (plus ampicillin 100µg/ml) and incubated for a few hours at 37°C with vigorous shaking (300rpm, Innova Incubator Shaker, New Brunswick Scientific, UK). The starter culture was diluted further (1 in 50) and shaken overnight at 37°C for a large scale plasmid preparation.

Bacterial cells were harvested 16h later by centrifugation (6000rpm, 15mins, 4°C, Sorvall RC SB, Survall Instruments, Connecticut, USA). The resulting cell pellet was resuspended in 10ml of Buffer P1, followed by the addition of 10ml of buffer P2. Contents were mixed by inversions and incubated at RT for 5min. 10ml chilled Buffer P3 was added and mixed by inversion before incubation on ice for 20min. The latter step allowed precipitation of proteins and chromosomal DNA from the bacterial cell suspension.

Cellular debris was removed by centrifugation (13,000rpm, 30min, 4°C; Sorvall RC SB). The resultant supernatant containing plasmid DNA was removed and recentrifuged to ensure complete removal of particulate debris. Plasmid DNA was further purified using the Qiagen-tip column. The column was equilibrated in 10ml Buffer QBT prior to usage. Supernatant was applied to the column under gravity and washed twice with 30ml Buffer QC. Plasmid DNA was eluted from the column in 15ml buffer QF. Plasmid DNA was precipitated by the addition of 0.7 volumes isopropanol and centrifuged promptly at 15,000g for 10min (Sorvall RC SB). The resultant DNA pellet was allowed to air-dry for 10min before the addition of Tris EDTA buffer, pH 8. Integrity of plasmid DNA was checked by agarose gel electrophoresis and concentrations calculated by spectroscopy (ratio 260/280nm), as follows:

**[(reading at 260nm) x (dilution factor) x 50] / 1000.**

### 2.6.3 Transient transfection of epithelial cell lines

In initial optimisation experiments three epithelial cell lines were utilised. Cells were seeded in 96-well plates at a density of 1 x $10^4$ cells /well in 200µl DMEM (for Caco-2 and HT-29) or MEM (for Hep2) complete media. Transfection was performed when cell monolayers reached 50-80% confluency. For transfection, FuGene reagent (Roche, Lewes, UK) in a ratio of 4:1 (DNA in µg : FuGene in µl) was used as recommended. Each transfection comprised a total DNA content of 230ng. This included:

a) **60ng**: Firefly luciferase construct under investigation, i.e. NFκB, hBD-2, -3 promoter, or over-expressing NOD 2 plasmids.
b) **20ng**: Renilla luciferase construct, to measure transfection efficiency and account for cell loss (only for luciferase reporter gene assay).
c) **150-170ng**: Empty vector (pcDNA) was added to equalise the total amount of DNA between all experiments.

A master mix of transfection reagent was prepared by adding FuGene (0.8µl/well) to low serum media (9.2 µl/well, Optimem, Invitrogen, Paisley, UK) and incubated at RT for a minimum of 15min. DNA was added and the mix incubated for a further 20min to allow DNA- Fugene complex formation, before the addition of 10µl of this master mix to each well. Negative controls (empty vector alone and no DNA) were also included. Transfections were allowed to proceed for 24h prior to cytokine or bacterial stimulation.

### 2.6.4 Luciferase reporter gene assay

Following transfection of luciferase and renilla reporter constructs and bacterial co-culture, cells were harvested by removing media followed by washes with ice-cold PBS. 50µl of 1 x passive lysis buffer (Promega, Southampton, UK) was added to each well. Plates were left at -80°C until required. For analysis, 20µl cell lysate was transferred to 2 luciferase assay plates. One plate was subjected to Renilla luciferase assay, where 40µl of its substrate, Coelentrazine (Insight Technologies, London, UK) was added to each well. 100µl firefly luciferase substrate (Promega, Southampton, UK) was added to each well in the second plate. IL-1β (20ng/ml), a potent agonist for IL-8 and hBD-2 gene expression was included as a positive control. A 96-well plate luminometer was utilised for measuring luciferase and renilla activity (LUCY1, Anthos Labtech Instruments, Salzburg, Austria). Promoter activity was analysed by normalising luciferase readings against renilla. Fold induction compared to non-stimulated control cells was deduced as follows:

$$\frac{\textbf{Stimulated cells: [(luciferase reading) / (renilla reading)]}}{\textbf{Non-Stimulated cells: [(luciferase reading) / (renilla reading)]}} = \textbf{Fold induction}$$

## 2.7 Statistics

Results are routinely presented as mean +/- Standard Error of Mean (+/-SEM). All experiments were conducted at least three times with transfection and colonisation assays performed in triplicate in each experiment. Statistical analyses were performed using GraphPad Instat statistical software, variables were compared using a *t* test, and a probability value of $<0.05$ was regarded as significant.

# CHAPTER 3

# Regulation of intestinal epithelial IL-8 and human β-defensins in response to *C. jejuni*

## 3.1 Introduction

The intestinal epithelium is formed by a monolayer of highly specialized cells, joint by tight junctions and covered by a thick mucus film. It is now widely accepted that it provides not only a physical barrier between the lumen and the underlying mucosa but also functions as a critical sensor for infections (Eckmann, 2006). Following specific sensing of a potential pathogen, the GI-epithelium is able to produce an array of innate defence molecules including cytokines, chemokines and antimicrobial peptides (Bevins *et al.*, 1999; O'Neil *et al.*, 1999; Ouellette, 2004; Eckmann and Kagnoff, 2005). Studies investigating the role of intestinal epithelial innate defence during *C. jejuni* infection are limited. Previous studies have shown induction of the proinflammatory cytokine, interleukin-8 (IL-8) by the intestinal epithelium in response to *C. jejuni* infection (Hickey *et al.*, 1999; Hickey *et al.*, 2000; Watson and Galan, 2005). IL-8 and other pro-inflammatory mediators are thought to be involved in initiating the host mucosal inflammatory response leading to diarrhoea and clearance of infection suggesting a role in disease pathogenesis (MacCallum *et al.*, 2006). Endogenous antimicrobial peptides such as the α- and β-defensin family, LL37 and lysozyme are known to be produced by the GI Paneth cells and epithelia. Evidence is accumulating suggesting a role for these peptides in GI host defence. However, the potential role of IEC antimicrobial peptides, specifically hBDs, in *C. jejuni* disease pathogenesis remains unknown. To gain a better understanding of IEC innate defence in response to *C. jejuni* we initiated studies investigating a potential role of hBDs during *C. jejuni* infection. Using two intestinal epithelium cell lines (HT-29 and Caco-2) as an *in-vitro* model, co-culture experiments with live WT *C. jejuni* strains 11168H and 81-176 were performed and hBD gene and protein expression was analysed by RT-PCR and Western blotting respectively. IL-8 gene expression was followed in parallel and used as a marker for infectivity in our model system. Furthermore, transcriptional regulation of IL-8, hBD-2 and hBD-3 was

also studied utilising their respective luciferase promoter constructs. As a known key regulator of innate gene transcription, an NF-κB promoter construct was included in our studies.

*C. jejuni* expresses several potential virulence determinants including flagellin mediated motility, adhesion and invasion of IECs (Wassenaar *et al.*, 1993; Hickey *et al.*, 1999; Zheng *et al.*, 2006). Additionally, the genome sequence of *C. jejuni* 11168 revealed the presence of a previously unsuspected capsular polysaccharide (CPS) locus that encodes a structure similar to the group II CPS described for *Escherichia coli* (Karlyshev *et al.*, 2000; Parkhill *et al.*, 2000b; Karlyshev *et al.*, 2001). The role of this capsule to date remains poorly defined. In order to gain further insight into the potential contribution of the bacterial capsule in eliciting host responses, an isgenic capsule mutant of *C. jejuni* strain 11168H (*kpsM*) was utilised in the present study.

## 3.2 Results

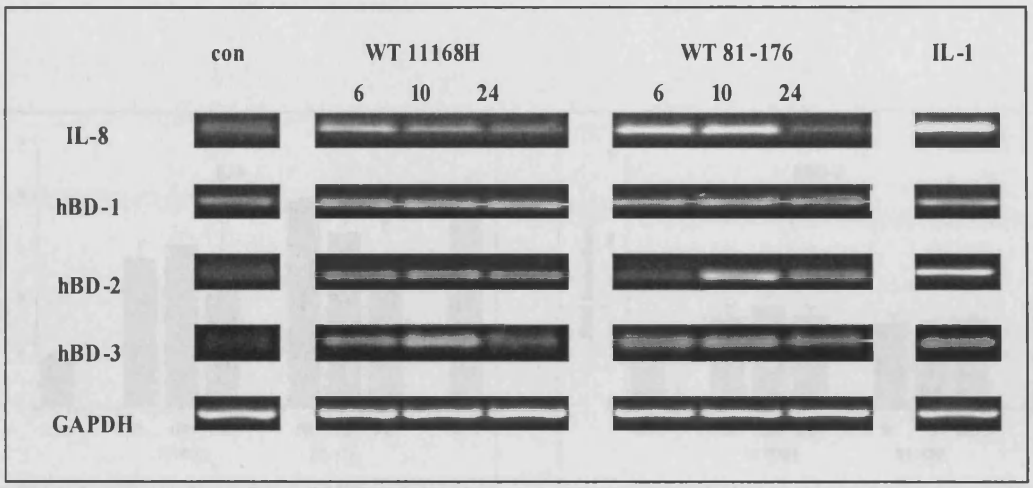
### 3.2.1 Modulation of IEC IL-8 and β-defensins gene expression during *C. jejuni* infection

IEC IL-8, hBD-1, hBD-2, and hBD-3 gene expression during infection with WT *C. jejuni* was investigated. Two virulent WT strains 81-176 and 11168H were chosen, which have been used frequently in other studies and shown to vary in their virulence and genetic set up (Bacon *et al.*, 2000; Ringoir and Korolik, 2003; Poly *et al.*, 2005). Co-culture experiments were performed over 24h, earlier innate immune events were monitored at 6h and 10h time points. Induction of IL-8 was observed in Caco-2 and HT-29 cells with two WT strains, these results were in agreement with previously published reports (Figure 3.1a and b, top lane). HBD-1 was found to be expressed in uninfected cells and no further modulation was observed during the 24h course of infection, suggesting no direct effect of *C. jejuni* (Figure 3.1a and b, second lane). HBD-2 and

hBD-3 expression was undetectable in uninfected control cells; however, both bacterial strains induced the expression of these two antimicrobial peptides in both cell lines (Figure 3.1a and b third and fourth lane). Similar to IL-8, induction of hBD-2 and hBD-3 mRNA levels was observed as early as 6h and induction persisted over the 24h time interval. No significant difference in IL-8, hBD-2 and hBD-3 gene regulation was observed between time points, bacterial strains or cell-line. However, induction of hBD-3 in response to both WT strains was found to be consistently modest in HT-29 when compared to Caco-2 cells (Figure 3.1a and b, fourth line).

In order to further investigate potential kinetic differences of innate gene regulation between the two bacterial strains, semi-quantitative analysis was performed using spot-densitometry. Data from two to three independent experiments were analyzed and average +/- SEM calculated. As shown in Figure 3.1c and d, constitutive expression of hBD-1 was confirmed with no further modulation observed in either cell line irrespective of WT strain utilised. Regulation of inducible innate genes was found to be similar for both WT strains and cell lines tested with an average of 2-3 fold increase in mRNA expression. Although, no significant differences were observed a trend for maximum gene induction of IL-8, hBD-2 and hBD-3 at 10h post-infection was noted (Figure 3.c and d). Further, WT strain 81-176 was found to be more potent in inducing innate gene expression compared to 11168H strain, however this did not reach statistical significance.

(a)




(b)

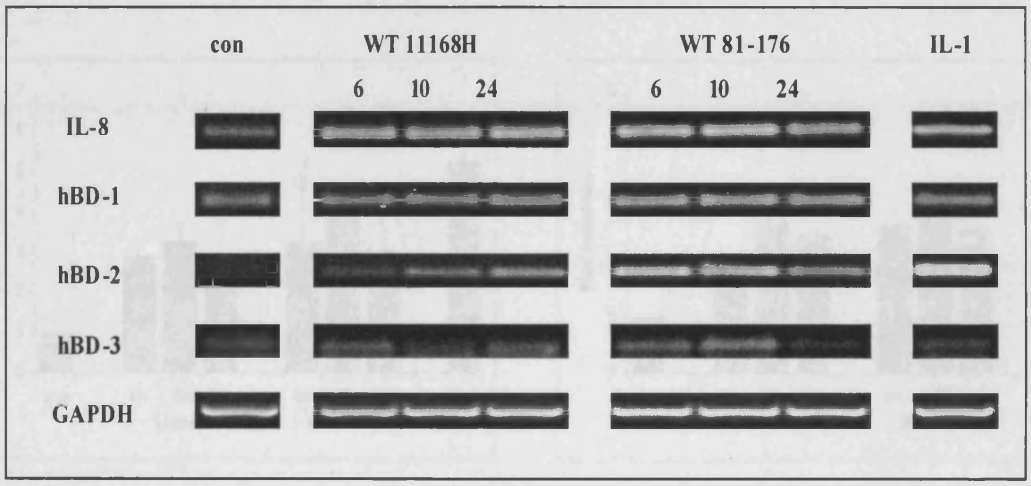



**Figure 3.1a and b: Modulation of IEC IL-8 and hBD gene expression during *C. jejuni* infection in Caco-2 (a) and HT-29 (b) cells.** Semi-confluent cells were infected with WT *C. jejuni* strain 11168H and 81-176 at an MOI of 100. Experiments were stopped after 6, 10 and 24h and innate gene expression analysed by RT-PCR. Un-infected cells and cells stimulated with IL-1β (20ng/ml) were used as negative and positive control, respectively. Experiments were performed at least 3 times, a representative gel is shown.

(c)

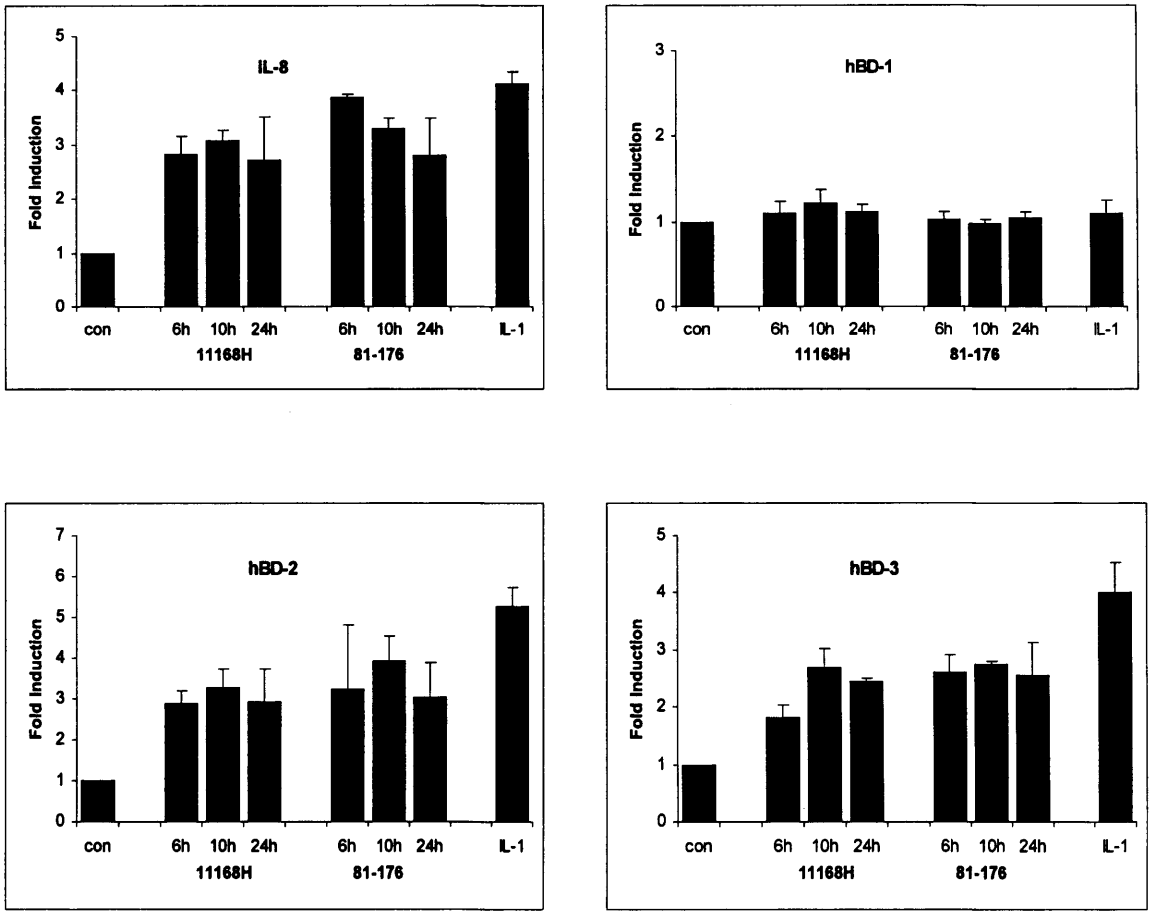



**Figure 3.1c: Semi-quantitative analysis of IL-8 and hBD gene expression during *C. jejuni* infection in Caco-2 cells.** Cells were infected with WT *C. jejuni* strains 11168H and 81-176. IL-8, hBD-1, hBD-2 and hBD-3 gene expression 6h, 10h and 24h post-infection was determined by RT-PCR. Gels were subjected to spot densitometric analysis and induction of mRNA levels presented as n-fold calculated compared to un-infected controls. Analysis of the housekeeping gene GAPDH was used to normalize gene expression. Data shown as mean (+/- SEM) induction of two to three independent experiments.

(d)

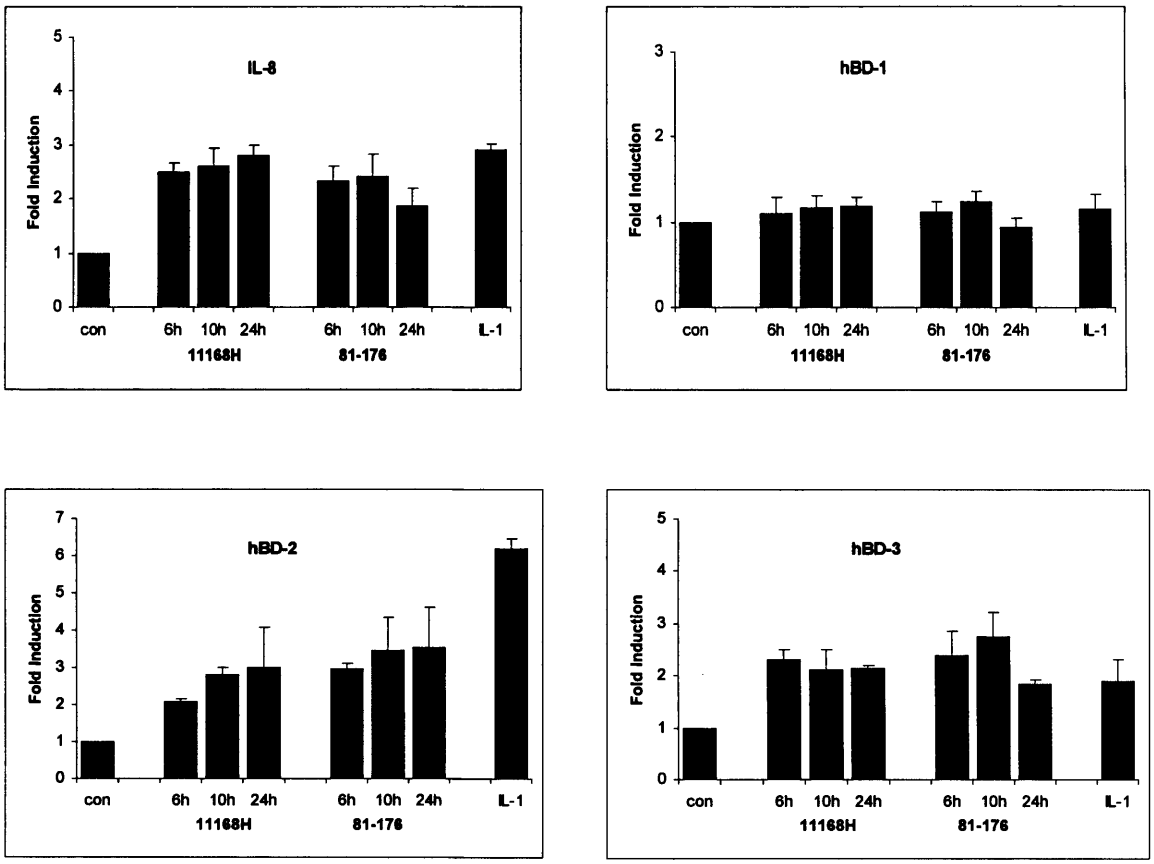



**Figure 3.1d: Semi-quantitative analysis of IL-8 and hBD gene expression during *C. jejuni* infection in HT-29 cells.** Data expressed as n-fold induction of mRNA levels compared to uninfected control cells. Error bars indicate mean (+/- SEM) induction of two to three independent experiments with similar results.

### 3.2.2 Induction of hBD-2 and hBD-3 peptide expression in response to *C. jejuni* infection

Expression of hBD-2 and hBD-3 peptides by IEC in response to *C. jejuni* was investigated. Caco-2 cells were infected with WT strain 11168H and Western blotting performed following 24h co-culture. In order to determine if hBDs are stored intracellularly or are mainly secretory in nature, both cell lysates and culture supernatants were analysed for the presence of peptide. Recombinant hBDs were used as positive controls. As shown in Figure 3.2 (top lane) hBD-2 was undetectable in uninfected controls (cells or supernatant), however the peptide was found to be present in the supernatant of cells infected with *C. jejuni*. Similarly, we observed undetectable levels of hBD-3 in un-stimulated cells (and supernatant, data not shown). However, the lysate of cells infected with *C. jejuni* 11168H was found to contain significant amounts of mature hBD-3 protein (Figure 3.2, bottom lane).

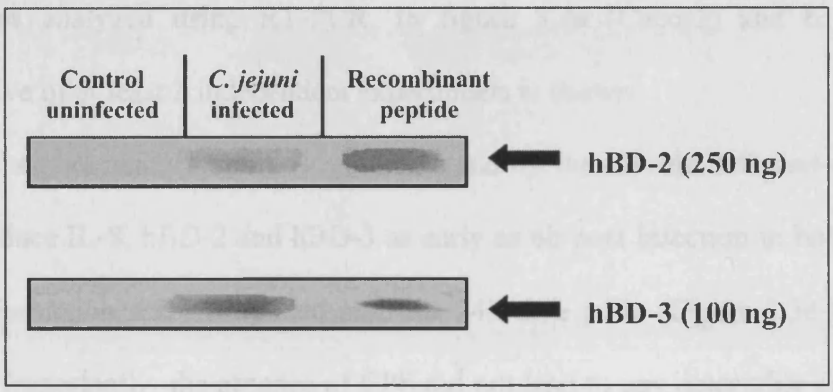


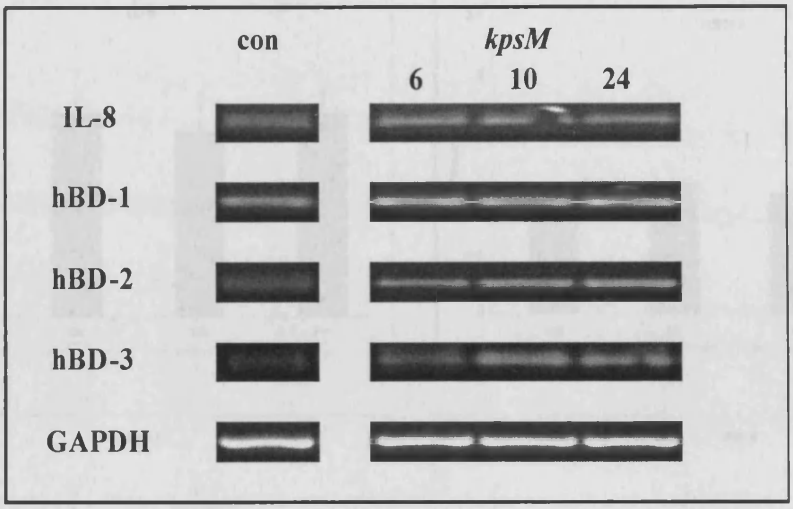

**Figure 3.2: HBD-2 and hBD-3 peptides are induced in response to *C. jejuni* 11168H.** Semi confluent Caco-2 cells were infected with WT *C. jejuni* strain 11168H for 24h. Culture supernatants (top lane) and cell lysates (bottom lane) were subjected to Tris-Tricine SDS-PAGE, followed by Western blotting. Recombinant peptides were included as positive control.

### 3.2.3 The role of *C. jejuni* capsular polysaccharide (CPS) in modulating IEC innate defence

An isogenic capsule deficient mutant of *C. jejuni* NCTC 11168H; *kpsM* was employed to investigate the role of CPS in modulating innate immune gene expression. As for WT bacteria, *kpsM* was grown on blood agar plates over 24h prior to use. Bacterial co-culture with Caco-2 and HT-29 cells was performed at an MOI of 100 and expression of innate genes analyzed using RT-PCR. In figure 3.3a (Caco-2) and b (HT-29) a representative of at least 3 independent experiments is shown.

Similar to the parental WT strain (Figure 3.1a and b), the capsule deficient mutant was found to induce IL-8, hBD-2 and hBD-3 as early as 6h post infection in both cell-lines tested and induction was maintained until the 24h time point (Figure 3.3a and b lanes 1,3 and 4). Importantly, the absence of CPS did not lead to any detectable difference in modulation of hBD-1, with expression remaining constitutive throughout the tested time interval (Figure 3.3a and b lane 2). Semi-quantitative analysis by spot densitometry suggested a 2 to 3-fold induction of IL-8, hBD-2 and hBD-3 by *kpsM* in both cell-lines (Figure 3.3c and d). No significant differences were observed between magnitude or kinetics of all innate genes tested when compared to parental WT strain 11168H (Figure 3.1c and d).

(a)




(b)

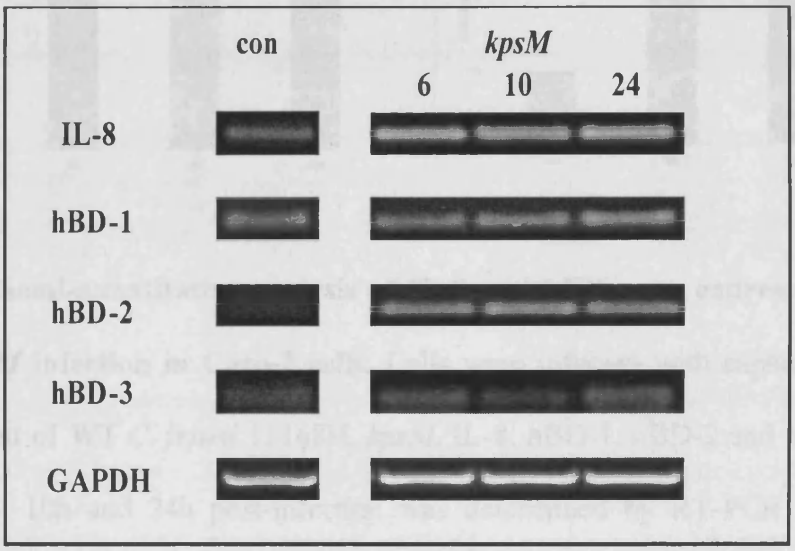



**Figure 3.3a and b: Modulation of IEC IL-8 and hBD gene expression by capsule deficient *C. jejuni kpsM* in Caco-2 (a) and HT-29 (b) cells.** Semi-confluent cells were infected with an isogenic capsule deficient mutant strain of *C. jejuni* strain 11168H, *kpsM* at an MOI of 100. Experiments were stopped after 6, 10 and 24h post infection, innate gene expression was analysed by RT-PCR. Un-infected cells and cells stimulated with IL-1β (20ng/ml) were used as negative and positive control, respectively. Experiments were performed at least 3 times, a representative gel is shown.

**(c)**

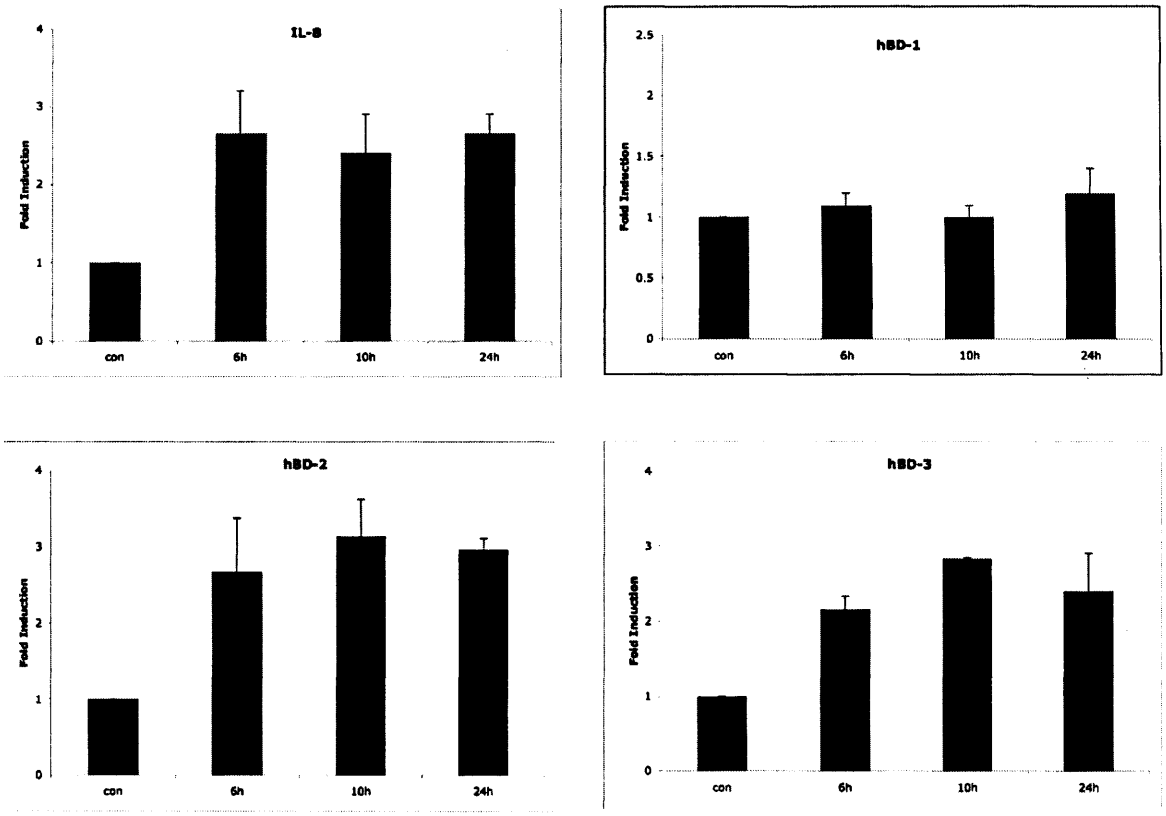



**Figure 3.3c: Semi-quantitative analysis of IL-8 and hBD gene expression during *C. jejuni kpsM* infection in Caco-2 cells.** Cells were infected with capsule deficient isogenic mutant of WT *C. jejuni* 11168H, *kpsM*. IL-8, hBD-1, hBD-2 and hBD-3 gene expression 6h, 10h and 24h post-infection was determined by RT-PCR. Gels were subjected to spot densitometric analysis and induction of mRNA levels calculated compared to un-infected controls. Housekeeping gene GAPDH was used to normalize gene expression. Increases in mRNA levels are expressed as n-fold induction compared to uninfected control cells. Data shown is mean (± SEM) induction of two to three independent experiments.

**(d)**

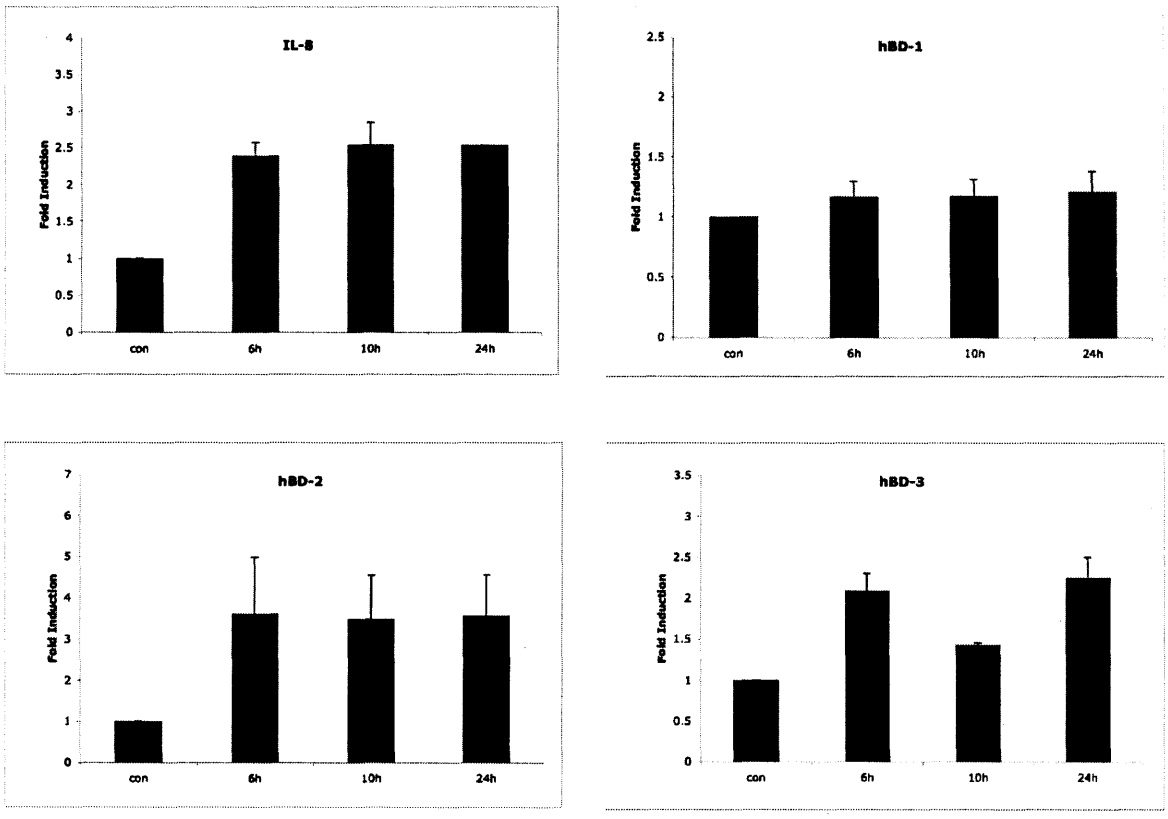



**Figure 3.3d: Semi-quantitative analysis of *C. jejuni kpsM* mediated modulation of IL-8 and hBD expression in HT-29 cells.** Spot-densitometry was performed and modulation of IL-8 and hBD expression in response to *C. jejuni kpsM* analysed. Results are presented as mean +/- SEM of two to three independent experiments.

### 3.2.4 IEC innate gene promoter studies during *C. jejuni* infection

Luciferase reporter gene assays are a powerful tool for molecular analysis of gene regulation. The promoter region of the gene of interest is cloned into a vector, which can then be introduced into target cells, by a mechanism referred to as transfection. With the promoter cloned upstream of luciferase coding sequence, appropriate promoter-activation should drive the expression of the luciferase enzyme. The subsequent measurement of luciferase enzymatic activity is therefore indicative of promoter function. Further, greater reliability is achieved by co-transfection of renilla, a vector that is expressed constitutively regardless of any stimuli. Inclusion of renilla vector provides a standard against which differences in cell numbers and transfection efficiency can be normalized. Finally, comparison of unstimulated versus infected cells allows quantification of the degree of promoter activation of the gene of interest. For greater detail notes by Promega Ltd. are recommended.

As RT-PCR only provided a semi-quantitative measure of IL-8 and β-defensin gene induction in response to *C. jejuni*, it was thought pertinent to investigate gene activation in greater detail. This was done by the inclusion of measurements of IL-8, hBD-2 and hBD-3 promoter-specific luciferase function. Furthermore, as a known central regulator of IEC innate gene transcription, an NF-κB promoter construct was also included in order to investigate potential differences of WT and mutant strains in their ability to modulate innate immune response.

#### 3.2.4.1 Method optimization

For initial optimization Hep2 cells were utilized mainly because this cell line exhibits high transfection efficiency. Various parameters (e.g. amount of DNA transfected, ratio of DNA to transfection reagent, cell numbers and time course) were optimized using NF-κB and IL-8 promoter constructs. A representative experiment is shown in Figure 3.4. Following transfection, cells were co-cultured with WT *C. jejuni* 11168H and 81-

176 strains over 6 and 20h. TNF-α, like IL-1β is a potent activator of IEC responses and was included as positive control. A 2 to 2.5-fold induction of IL-8 and NF-κB promoter activity was noted in response to both WT strains (Figure 3.4a). A maximum of 3.5-fold induction after stimulation with TNF-α was observed both at 6 and 20h.

**(a)**

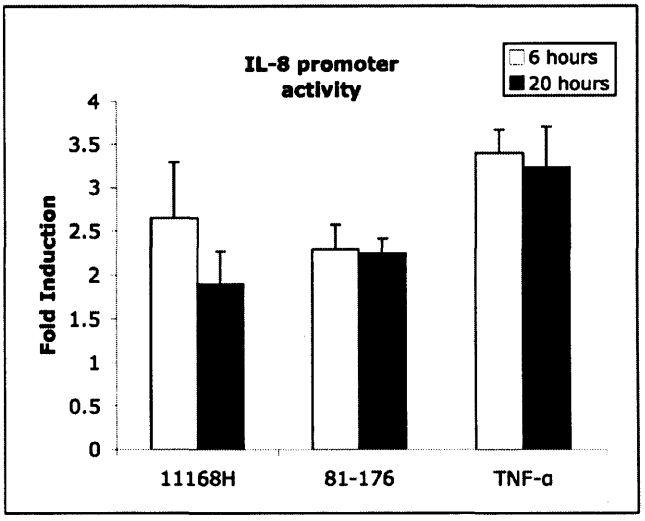



**Figure 3.4a: Optimization of IL-8 promoter activity in response to *C. jejuni* in Hep2 cell line.** Hep2 cells were transiently transfected over 20h followed by co-culture with WT *C. jejuni* strains 11168H and 81-176 for 6 and 24h. Cells stimulated with TNF-α were included as positive controls. Following stimulation, luciferase activity was measured and individual readings normalized against renilla. Fold induction was calculated compared to unstimulated control cells. Experiments were performed two to three times in triplicates. A representative experiment is shown; arrow bars indicate average +/- SEM of triplicate values.

Following optimization in Hep2 cells, experiments were conducted in intestinal epithelial HT-29 and Caco-2 cell-lines. Dose dependant induction of IL-8 promoter activity by WT *C. jejuni* 11168H was found after 20h (Figure 3.4b). IL-1β, a potent agonist for IL-8 and hBD2 induction was routinely included as a positive control. Amongst the two cell-lines tested, Caco-2 cells consistently showed greater and more reliable transfection efficiency with higher promoter activity when compared to HT-29 cells (Figure 3.4b). For this reason subsequent studies were performed in Caco-2 cells.

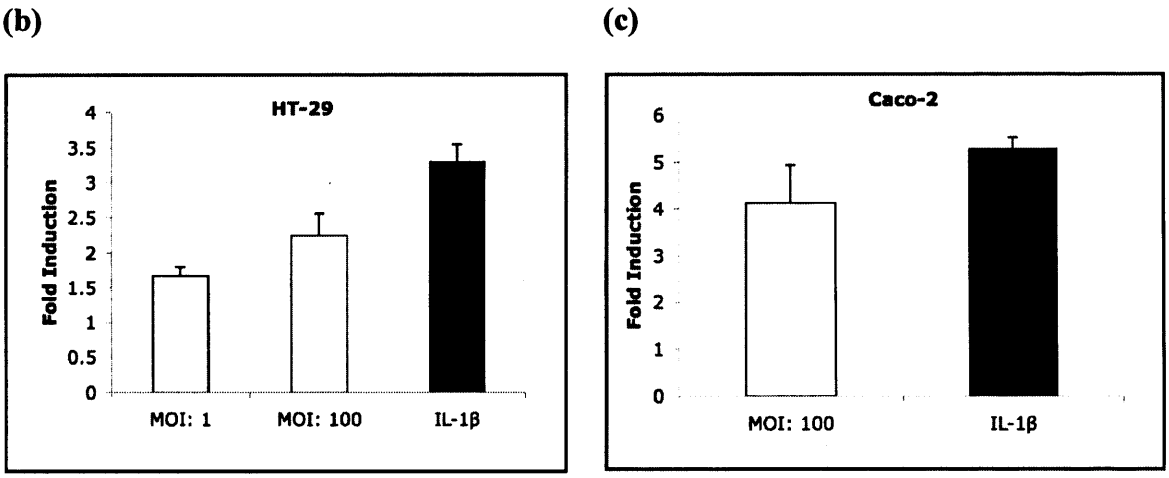


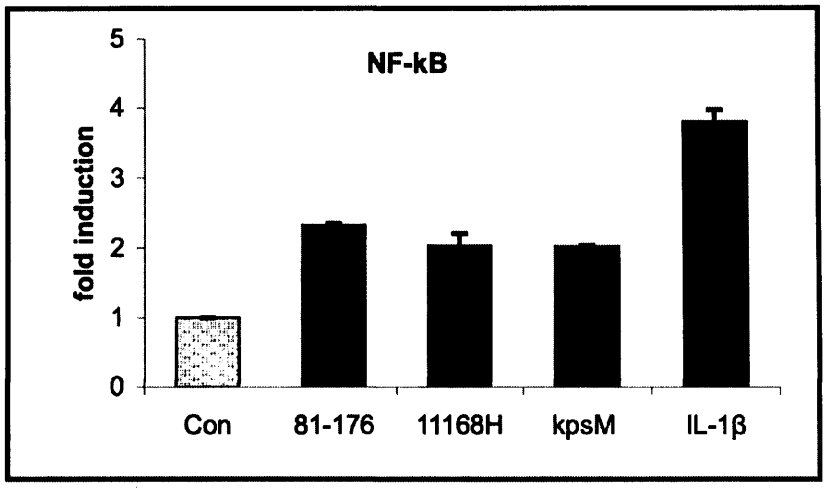

**Figure 3.4b and c: Optimization of IEC IL-8 promoter activity in response to *C. jejuni*.** HT-29 (b) and Caco-2 (c) cells were transiently transfected over 20h followed by co-culture with WT *C. jejuni* strain 11168H for 20h. Infection was performed at an MOI of 1 and 100 in HT-29 and MOI of 100 in Caco-2 cells. Cells stimulated with IL-1β served as a positive control. Luciferase activity was normalized against renilla and fold induction calculated compared to unstimulated control cells. A representative of 2 to 3 independent experiments is shown; arrow bars indicate average +/- SEM of triplicate values.

3.2.4.2 Regulation of innate immune gene promoter activity by *C. jejuni*

Following optimization, the ability of WT *C. jejuni* 81-176, 11168H and capsule deficient isogenic mutant *kpsM* to regulate innate immune genes was investigated. NF-κB and IL-8 promoter constructs were transiently transfected into Caco-2 cells followed by bacterial co-culture over 20h. As shown in Figure 3.4d, two to three-fold induction of NF-κB promoter activity was observed for all bacterial strains tested, with no significant differences noted between WT or mutant strain. Induction of IL-8 promoter was also found to be similar for all bacterial strains tested (Figure 3.4e). Stimulation with IL-1β led to 4-fold induction of both NF-κB and IL-8 (Figure 3.4d and e). Initial time course experiments revealed no significant differences in promoter activities, however induction was found to be most consistent after 20h co-culture (data not shown).

Regulation of hBD-2 and hBD-3 promoter activity by *C. jejuni* was also investigated. Similar to NF-κB and IL-8, WT *C. jejuni* caused two to three fold induction of both hBD-2 (Figure 3.4f) and hBD-3 (Figure 3.4g) promoters with the latter one providing a more modest response. The *kpsM* mutant was found to be equally potent in inducing hBD-2 and hBD-3 promoter activity.

(d)




(e)

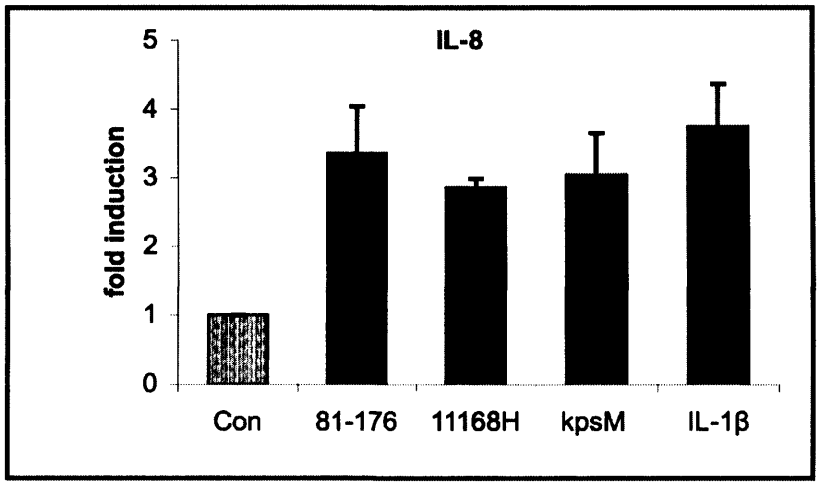
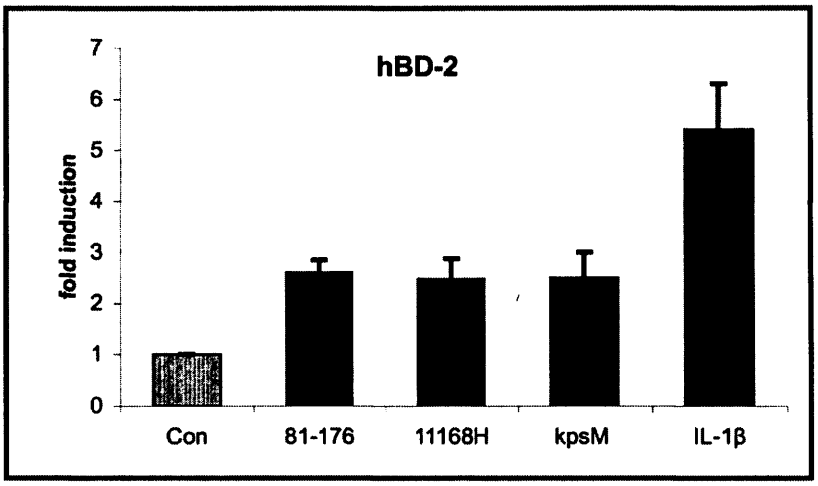



**Figure 3.4d and e: NF-κB and IL-8 promoter activity in response to *C. jejuni*.** Caco-2 cells were transiently transfected with NF-κB and IL-8 promoter constructs over 24h. Bacterial co-culture with WT *C. jejuni* strains 81-176, 11168H and isogenic capsule deficient *kpsM* was performed at MOI of 100 for 20h. Cells stimulated with IL-1β (20ng/ml) were included as positive control. Luciferase activity was measured and individual readings normalized against renilla. Promoter activity is expressed as n-fold induction compared to unstimulated control cells. Arrow bars indicate average +/- SEM of three independent experiments performed in triplicate.

(f)




(g)

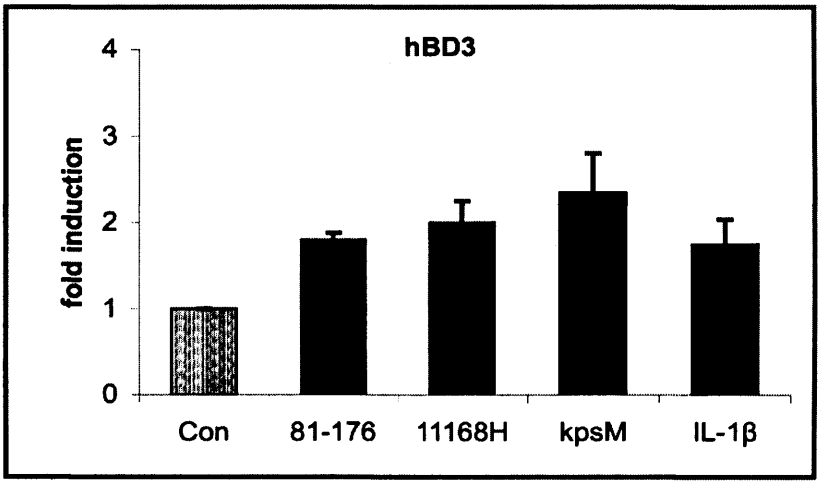



**Figure 3.4f and g: HBD-2 and hBD-3 promoter activity in response to *C. jejuni*.** Caco-2 cells were transiently transfected with hBD-2 and hBD-3 promoter constructs over 24h. Co-culture with WT *C. jejuni* strains 81-176, 11168H and isogenic capsule deficient *kpsM* was carried out for 20h at MOI of 100. Cells stimulated with IL-1β (20ng/ml) served as positive control. Luciferase activity was measured and individual readings normalized against renilla. Promoter activity is expressed as n-fold induction compared to unstimulated control cells. Arrow bars indicate average +/- SEM of three independent experiments performed in triplicate.

## 3.3 Discussion

*C. jejuni* is a commensal organism of poultry but represents a major health burden in humans. Poultry are routinely colonized with up to 1000 *C. jejuni* cells per g of cecal contents in an apparently commensal association that causes little or no pathology (Shreeve *et al.*, 2000). In contrast, as few as 800 *C. jejuni* cells are sufficient to cause severe inflammatory gastroenteritis in human volunteer studies (Black *et al.*, 1988). The bacterium causes a spectrum of clinical diseases, yet in the majority of healthy individuals, the infection is short lived and self limiting, suggesting an important role for innate immunity in detecting and clearing the infective agent. The intestinal epithelium as the first line of defence not only provides a physical barrier to the underlying mucosa but also produces several innate defence molecules upon microbial sensing, such as the pro-inflammatory cytokine IL-8. Several studies have demonstrated secretion of IL-8 by IEC in response to *C. jejuni* and this mechanism is thought to play an important role in initiating the inflammatory response observed *in-vivo* (Hickey *et al.*, 1999; Hickey *et al.*, 2000; Watson and Galan, 2005). As a second part of host innate defence, host epithelial antimicrobial peptides have been shown to contribute to host defence at mucosal surfaces (Ganz, 2003; Salzman *et al.*, 2003; Dommett *et al.*, 2005). Dynamic modulation of β-defensins has been demonstrated with various models of GI infection and inflammation (O'Neil *et al.*, 1999; Bajaj-Elliott *et al.*, 2002; Wehkamp *et al.*, 2003a). However, to date, the role of these peptides during *C. jejuni* infection remains unknown.

The aim of this part of the study was to investigate the regulation of IEC β-defensins during *C. jejuni* infection. Expression of IL-8 was also investigated in parallel, primarily as it served as an indicator for degree of infectivity in our cell line model. Using two IEC-lines (Caco-2 and HT-29), we demonstrated constitutive expression of hBD-1. Infection with two WT strains of *C. jejuni* 11168H and 81-176 did not have any

effect on hBD-1 gene expression in either cell line over a 24h time course, suggesting that *C. jejuni* does not regulate expression of this AMP (Figure 3.1a and b, lane 2). In contrast, uninfected IECs did not express hBD-2 or hBD-3, but the presence of *C. jejuni* resulted in increased expression as early as 6h post infection (Figure 3.1a and b, lanes 3 and 4). This data highlights the ability of IECs to directly mount a fast antimicrobial response to the presence of *C. jejuni*. In agreement with other studies we confirmed induction of IL-8 by both WT strains in Caco-2 and HT-29 cells (Figure 3.1a and b, lane 2). Induction of all innate genes tested was found to follow similar kinetics with induction observed as early as 6h and still maintained 24h post-infection, suggesting overlapping signalling pathway(s) are likely to be responsible for early host innate defence. Despite the lack of statistical significance, the magnitude of this response was found to be consistently greater with the WT 81-176 strain compared to 11168H. It has previously been shown that different *C. jejuni* strains elicit different levels of IL-8, this response maybe associated with the invasive potential of the bacterium under study (Hu and Hickey, 2005; Watson and Galan, 2005). Differences in the invasive capacity of 81-176 and 11168H might therefore explain the observed differences in innate gene activation. Furthermore, *C. jejuni* strain 81-176 has been shown to harbour a plasmid pVir, which has been linked to bacterial virulence. This plasmid is absent in strain 11168H providing an alternative/additional explanation for our findings (Bacon *et al.*, 2000; Ringoir and Korolik, 2003; Hu *et al.*, 2006a). Data obtained by RT-PCR was confirmed and further extended using promoter gene studies. We observed an average 2 to 3-fold induction of NF-κB, IL-8, hBD-2 and hBD-3 promoter activity by both WT strains (Figure 3.4d-g).

Investigation of hBD-2 and hBD-3 protein expression in response to WT *C. jejuni* 11168H confirmed correlation between innate gene and protein expression of both antimicrobial peptides (Figure 3.2). Additionally, hBD-2 was recovered from culture

supernatant, confirming the secretory nature of this peptide (Sorensen *et al.*, 2005; Kumar *et al.*, 2006). In contrast, hBD-3 was found in the cellular fraction. One may hypothesize that the differential compartmentalization of hBD-2 and hBD-3 may represent different frontiers of host defence, i.e. hBD-2 targeting adhering, extracellular bacteria, whereas hBD-3 might act on invading organisms. In accordance with these findings, a similar differential distribution of hBD-2 and hBD-3 was recently reported in a skin model of infection and inflammation (Sorensen *et al.*, 2005).

Bacterial CPS has been reported to play a role in modulating host immune response. Investigation into the role of *C. jejuni* CPS in IEC responses surprisingly showed that the capsule-deficient isogenic *kpsM* mutant modulated IL-8 and β-defensins gene expression to a similar extent to that observed for the 11168H parental strain (Figures 3.3 and 3.4).

In summary, these studies confirmed the ability of WT *C. jejuni* to induce expression of IL-8 and NF-κB. However, this is the first study to highlight increase of hBD-2 and hBD-3 gene and protein expression in response to WT strains 81-176, 11168H and the capsule deficient mutant *kpsM.*

To date, mechanism(s) implicated in *C. jejuni* mediated IEC IL-8 induction include bacterial adherence and/or invasion, de-novo protein synthesis and the presence of CDT (Hickey *et al.*, 2000; Watson and Galan, 2005). An 81-176 *kpsM* mutant has previously been shown to have a reduced ability to invade INT407 cells (Bacon *et al.*, 2001). This suggests that if the 11168H *kpsM* mutant employed in our study also has reduced ability to invade intestinal epithelial cells, then induction of IL-8 by this mutant may occur primarily via CDT-dependant mechanism. The invasive capacities of all strains used in this study are currently being investigated in a collaborative study (A. Elmi, N. Dorrell, B. Wren, unpublished data). However, even reduced levels of invasion might be sufficient to induce IL-8 and β-defensin expression. This is supported by recent studies

showing that despite an association between IL-8 induction and invasion, there seems to be no direct correlation. This aspect will be addressed in more detail in chapter 5.

In order for β-defensins to play a major role in disease pathogenesis it is not sufficient for these petides to be up-regulated early during the course of infection, but also to be capable of inactivating and/or killing the pathogen. The bactericidal potency of β-defensins against *C. jejuni* was therefore investigated as described in the following chapter.

# CHAPTER 4

# Susceptibility of *C. jejuni* to human β- defensins

## 4.1 Introduction

It is becoming increasingly evident that β-defensins like chemokines are multifunctional molecules with a wide range of properties (Ganz, 2003; Eckmann, 2005). What distinguishes these cationic peptides from other innate defence molecules is their ability to directly cause damage and ultimately kill pathogens. These peptides exhibit antimicrobial activity against a range of micro-organisms including Gram-positive and Gram-negative bacteria, fungi, protozoa and even enveloped viruses (Ganz, 2003; Agerberth and Gudmundsson, 2006; Dann and Eckmann, 2007).

We demonstrated induction of IEC hBD-2 and hBD-3 gene and peptide expression in response to *C. jejuni* infection (Chapter 3) suggesting a potential involvement of these molecules in disease pathogenesis. However, in order to play an important role in limiting bacterial colonisation and ultimately clearing infection, β-defensins must exhibit bactericidal activity against the bacterium. The aim of this part of the study was therefore to investigate the susceptibility of *C. jejuni* to antimicrobial peptides known to be expressed by the IEC. These include hBD-1, -2 and -3 and lysozyme, a ubiquitous AMP known to be expressed constitutively in the upper GI tract and small intestine (Montero and Erlandsen, 1978). The potential role of IEC LL-37 in *C. jejuni* disease pathogenesis was not investigated in the present study.

Bacterial CPSs have been shown to mediate protection against the bactericidal activity of AMPs. For example CPS of *Klebsiella pneumoniae* protects the organism against host innate immune defence by limiting interaction of AMPs with the bacterial membrane (Campos *et al.*, 2004). Although *C. jejuni* CPS did not contribute to intestinal innate defence and hBD expression (Chapter 3), it's primary function maybe to provide a protective shield against the antimicrobial action of hBDs or other AMPs (i.e. lysozyme).

In order to investigate the above hypothesis, a bactericidal assay was established. Briefly, *C. jejuni* bacterial cells were exposed to recombinant hBDs and lysozyme over various time periods. Bactericidal activity was assessed by plating out serial dilutions and quantifying viable counts 2-3 days later. We also employed Scanning Electron Microscopy (SEM) to further our understanding of structural changes to *C. jejuni* caused by β-defensin action.

## 4.2 Methods

### 4.2.1 Bactericidal assay

*C. jejuni* was routinely grown on blood agar plates over 24h (mixed-phase culture) followed by resuspension and a further 16h incubation in MH broth preformed to obtain mid-log phase culture. Prior to assay, bacteria were resuspended in 10mM phosphate buffer pH 7.4 and number of bacteria quantified. Approximately 1 x $10^5$ CFU/ml organisms were exposed to recombinant hBDs and lysozyme for indicated time periods at 37°C under microaerophilic conditions. *C. jejuni* in buffer alone was included to establish and distinguish bacterial survival rate in low salt conditions *versus* survival in the presence of defensin peptides. Experiments were terminated by plating serial dilutions of the reaction mix in triplicates onto blood agar plates. Bactericidal activity was accessed by counting viable bacteria after 2 to 3 days.

### 4.2.2 Scanning Electron Microscopy (SEM)

Firstly, a bactericidal assay was performed, exposing WT *C. jejuni* (NCTC 11168H) to recombinant hBD-3 ($10^{-6}$ M) for 30min. Prior to this, silicon wafers were prepared by immersion in 0.1% (v/v) poly-L-Lysine (Sigma-Aldrich, UK) for 15min followed by air drying for 30min. 100µl of hBD-3 exposed bacterial suspension was adsorbed onto wafer for 15min. Bacteria coated wafers were immersed in 0.5 % (v/v) glutaraldehyde

(Sigma-Aldrich, UK) in PBS for 5min at ambient temperature and stored in PBS at 4°C until further processing.

The following steps were performed by Mr. K. Pell (Department of Biological Sciences, University of London at the Queen Mary's School of Medicine and Dentistry, London UK). Wafers were washed in distilled water prior to immersion in 1% (w/v) osmium tetroxide (Sigma-Aldrich, UK) for 15min. Following a second series of three washes in distilled water, the wafers were passed through a series of ascending concentrations of ethanol (30%, 50%, 70%, and 90%) washes for 5min each followed by two washes in absolute ethanol for 10min. Wafers were then dried using absolute ethanol as transfer fluid in a BALZER'S CPD 030 critical point drier (Bal-Tec, Liechtenstein). The wafers were finally mounted on carbon supports, sputter coated with gold using a BALZER'S SCD 030 sputter coater (Bal-Tec, Liechtenstein), and examined in a JEM 1200EXII scanning transmission electron microscope (Jeol, Tokyo, Japan) operating in the scanning mode, between 40 and 60 kV.

## 4.3 Results

### 4.3.1 Recombinant hBD-2 and hBD-3 exhibit potent bactericidal activity against WT *C. jejuni* NCTC 11168H

Firstly, bactericidal activity of hBD-1, hBD-2 and hBD-3 against WT *C. jejuni* 11168H was tested. Approximately $1x10^5$ CFU of a 24h bacterial culture (i.e. colony from blood agar plates) was exposed to $10^{-6}$M recombinant peptides for 30 or 60min prior to streaking out serial dilutions. Time course and concentration of recombinant peptides were chosen according to preliminary experiments and previous work undertaken in our laboratory (George *et al.*, 2003). Bactericidal activity was calculated as the percentage of viable counts obtained in buffer alone minus those exposed to recombinant peptides. Data is shown as an average of 3 independent experiments (preformed in triplicates) and

error bars indicating +/-SEM. As shown in Figure 4.1, hBD-2 and hBD-3 exhibited potent bactericidal activity, with more than 99% of bacteria killed after 30min exposure to peptides. In contrast, the constitutively expressed hBD-1 was found to be significantly less potent and more variable (large SEM), its bactericidal activity ranging from 0 to 20%. Interestingly, we noted increased survival of bacteria exposed to hBD-1 for 60min compared to 30min. However, this effect was not statistically significant.

With bactericidal activity of both inducible hBD-2 and hBD-3 reaching almost 100% at a concentration of $10^{-6}$M, peptides were further diluted in order to investigate differences in their potency and establish a minimal concentration required for killing. As shown in Figure 4.2, hBD-3 exhibited a slightly higher bactericidal activity at concentrations tested when compared to hBD-2. Both hBD-2 and hBD-3 proved to be highly efficient at $10^{-7}$M, rendering >99% of bacteria nonviable. The minimal concentration required for killing was found to be $5x10^{-8}$M for both peptides. Interestingly, lower concentrations did not demonstrate any bactericidal effect, highlighting an "all or none" mode of action. The observed potent bactericidal activity of hBD-3 led us to further investigate kinetics of hBD-3 mediated *C. jejuni* killing. As shown in Figure 4.3 following only a 5min exposure, $10^{-7}$M hBD-3 killed more than 99.5% of bacteria, suggesting rapid killing kinetics of this AMP against *C. jejuni*.

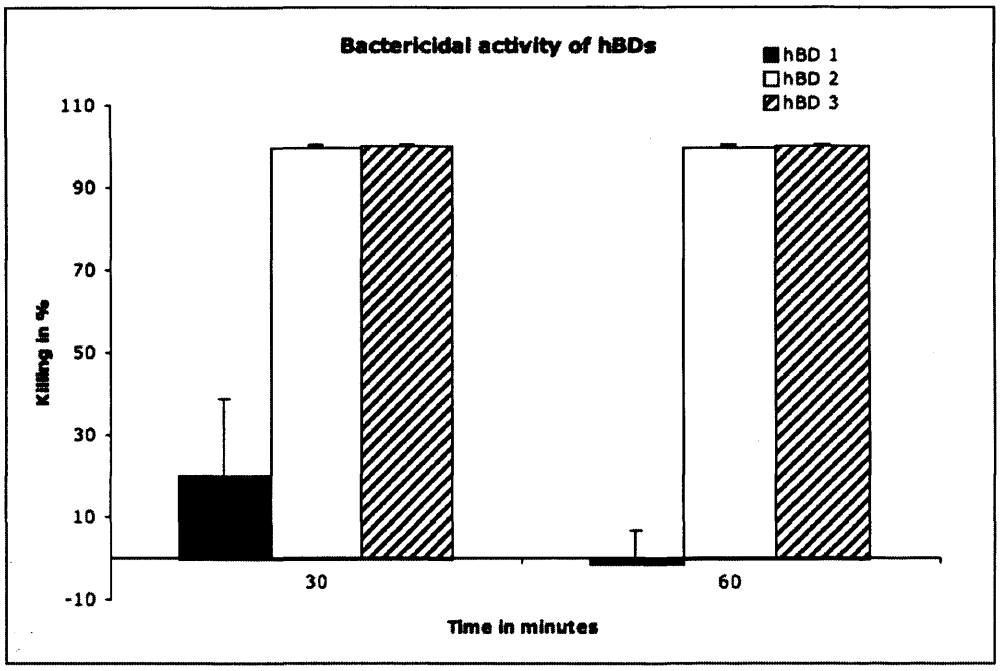



**Figure 4.1: Bactericidal activity of recombinant hBDs against WT *C. jejuni* strain 11168H.** $10^5$ CFU/ml *C. jejuni* were exposed to $10^{-6}$M hBD-1, hBD-2 and hBD-3 in 10mM phosphate buffer. Viability was assessed after 30 and 60min exposure. Bactericidal activity was calculated against control samples containing bacteria in buffer alone. Data represents the mean +/-SEM of 3 independent experiments, each performed in triplicate.

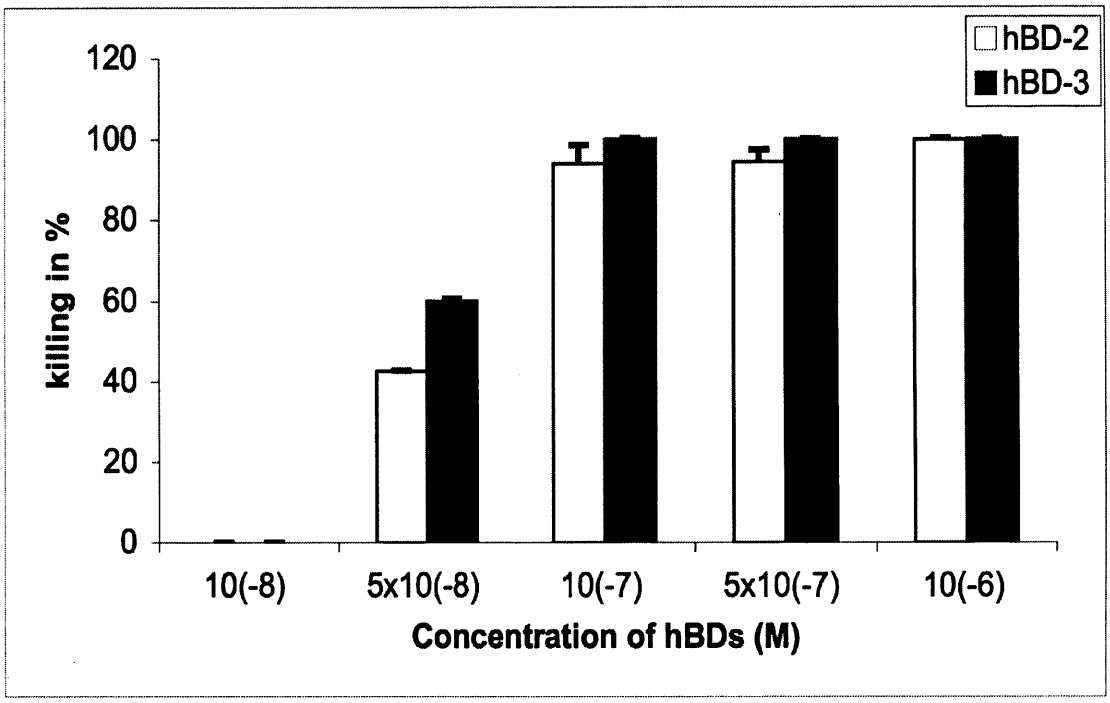



**Figure 4.2: Dose dependent bactericidal activity of hBD-2 and hBD-3 against WT *C. jejuni* strain 11168H.** *C. jejuni* WT 11168H was incubated with recombinant hBD-2 and hBD-3 in a $10^{-6}$ to $10^{-8}$M concentration range for 30min and bactericidal activity calculated. Data represents mean +/-SEM of three experiments, each performed in triplicate.

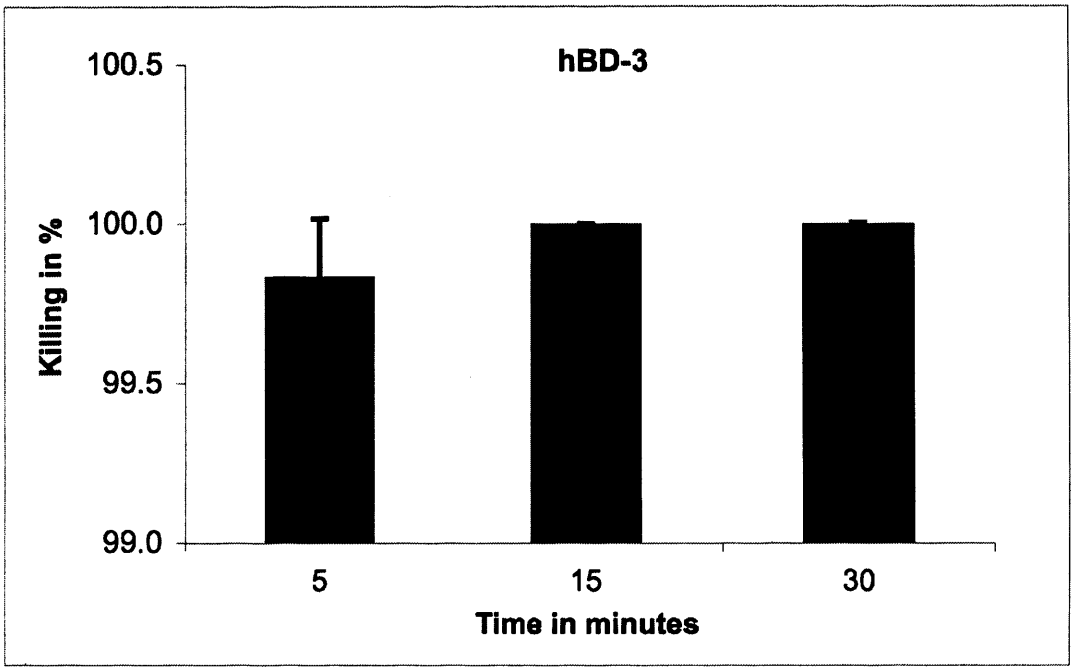



**Figure 4.3: Kinetics of antimicrobial activity of hBD-3 against WT *C. jejuni* 11168H.** *C. jejuni* WT 11168H was incubated with $10^{-7}$M hBD-3 and serial dilutions plated out on blood agar after 5, 15 and 30min. Data represents the mean +/-SEM of three independent experiments, each performed in triplicate.

### 4.3.2 Effect of bacterial growth phase on susceptibility of *C. jejuni* to hBDs

Bacterial growth phase is known to be associated with changes in expression of various surface structures of *C. jejuni* including capsular polysaccharide and changes in metabolic activity (Bacon *et al.*, 2001; Karlyshev *et al.*, 2002; Vandecasteele *et al.*, 2004). These changes can influence resistance to the action of antibiotics and antimicrobial peptides (McLeod and Spector, 1996; Kobayashi *et al.*, 2006). We investigated the impact of *C. jejuni* growth phase on its susceptibility to the antimicrobial action of hBDs. Bacteria grown on blood agar plates for 24h represented a mixed population of organisms, whereas *C. jejuni* grown further in Muller Hinton broth for 16h were considered mid-logarithmic (mid-log) phase bacteria, the latter representing dividing, metabolically active organisms. As shown in Figure 4.4, no significant difference in the susceptibility to the antimicrobial action of $10^{-6}$M hBD-2 and hBD-3 was observed between mid-log and mixed phase bacterial cultures. Interestingly, an average difference of approximately 20% higher killing of mid-log phase bacteria by hBD-1 was observed, suggesting actively dividing bacteria might be more susceptible to hBD-1. However, due to large variability (expressed in large +/- SEM) between experiments this difference was found not to be statistically significant. Further dilutions might be necessary to investigate if these potential differences are real.

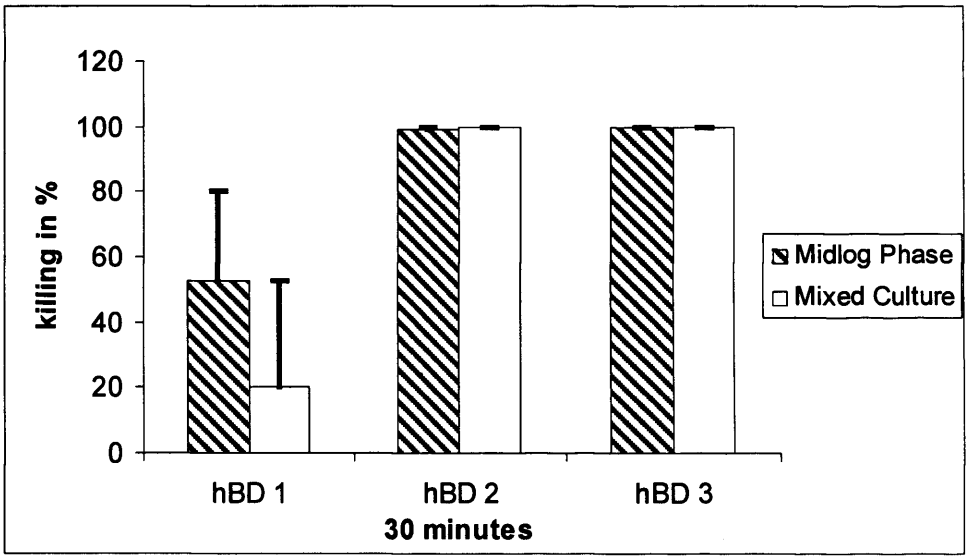



**Figure 4.4: Impact of bacterial growth phase on susceptibility to the antimicrobial activity of hBDs.** $10^5$ CFU/ml of mid-log phase and mixed cultured *C. jejuni* 11168H were incubated in $10^{-6}$M hBDs for 30min prior to serial dilution plating. Data are shown as mean +/- SEM of three independent experiments, each performed in triplicate.

### 4.3.3 Bactericidal activity of lysozyme against *C. jejuni*

Another epithelial antimicrobial peptide known to be expressed in the GI tract is lysozyme. Firstly, we investigated expression of lysozyme in our tissue culture model during a 24h infection with WT *C. jejuni* 11168H. As shown in Figure 4.5, we found constitutive lysozyme expression in control Caco-2 cells with no further modulation by *C. jejuni* noted during 24h of infection (Figure 4.5 middle lane). Infectivity was confirmed by following induction of IL-8 in the same experiment (top lane).

Having confirmed the presence of lysozyme in our model system, we wished to test the bactericidal activity of lysozyme against WT *C. jejuni* 11168H. Further, we also investigated any potential synergistic effects between lysozyme and hBD-1, as both peptides are constitutively expressed and are likely to be present at the same time at the GI mucosal surface. As shown in Figure 4.6, 1.4μM (20μg/ml) lysozyme and $10^{-6}$M hBD-1 exhibited a similar range of bactericidal activity against *C. jejuni*. Interestingly, combining both AMP in equal concentrations lead to increased bactericidal activity, suggesting an additive and/or synergistic effect (Figure 4.6 left column).

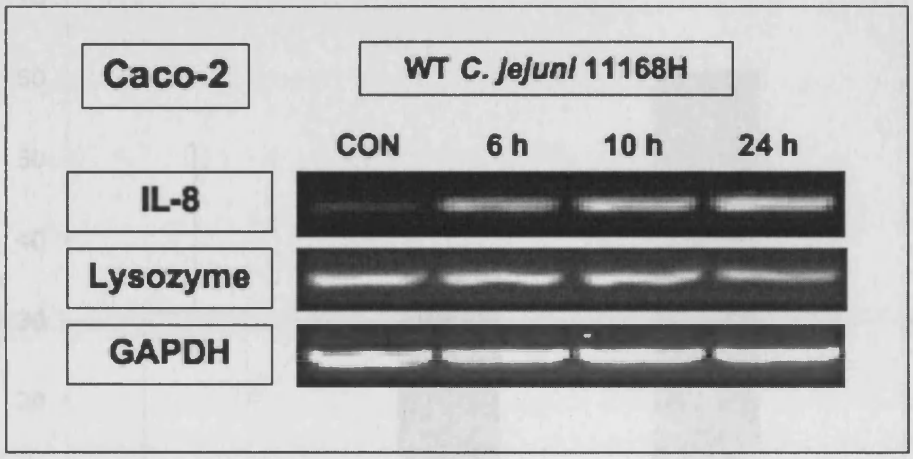



**Figure 4.5: Expression of lysozyme in Caco-2 cells infected with WT *C. jejuni* strain 11168H.** Caco-2 cells were co-cultured with WT *C. jejuni* strain 11168H at an MOI of 100. Lysozyme and IL-8 gene-expression was followed by RT-PCR after 6, 10 and 24h and normalized against GAPDH. Uninfected cells served as control. A representative gel of three independent experiments is shown.

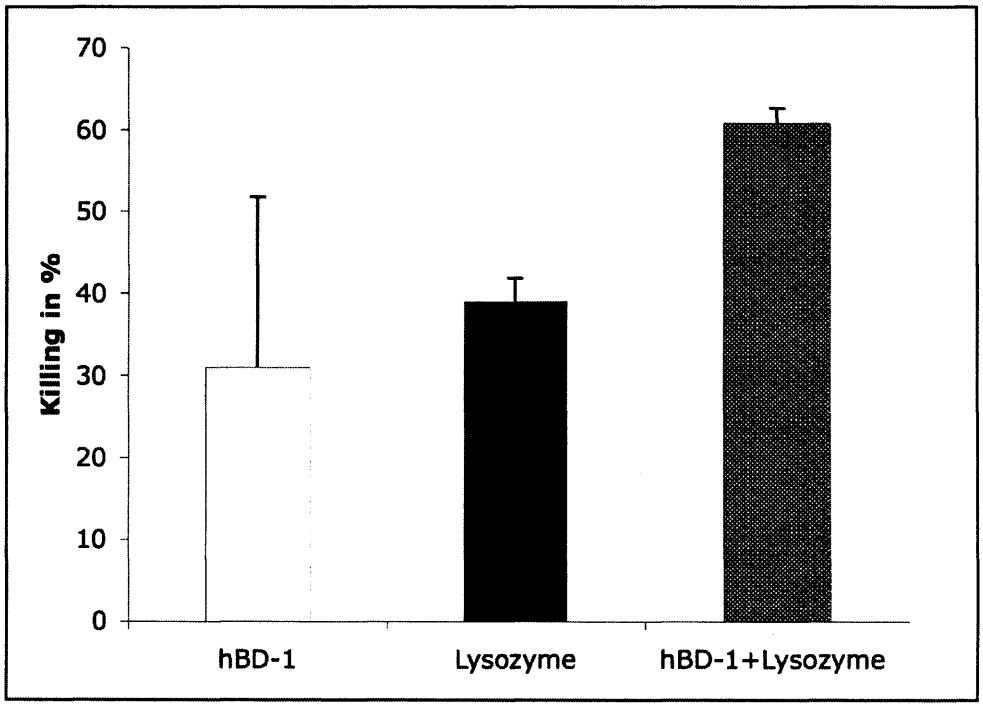



**Figure 4.6: Bactericidal activity of the constitutively expressed antimicrobial peptides hBD-1 and lysozyme against *C. jejuni*.** WT *C. jejuni* 11168H was incubated with either $10^{-6}$M hBD-1, $1.4x10^{-5}$M (20μg/ml) lysozyme alone or both peptides combined for 30min. Data represents an average +/- SEM of two to three independent experiments, each performed in triplicate.

### 4.3.4 The role of *C. jejuni* capsular polysaccharide in protection against AMPs

Studies illustrated in chapter 3 revealed that the capsule deficient isogenic mutant strain of *C. jejuni* WT 11168H was equally potent in inducing IL-8, hBD-2 and hBD-3 expression suggesting a minor role of this structure in modulating host epithelial innate immune defence. However, it has been shown that bacterial capsule is protective against antimicrobial peptides in other Gram-negative bacteria (Campos *et al.*, 2004). To investigate the potential role of *C. jejuni* CPS in protecting the organism against AMPs, *kpsM* was subjected to bactericidal assays using recombinant hBDs and lysozyme. As shown in Figure 4.7 hBD-2 and hBD-3 were equally potent against WT and *kpsM*. In contrast there was a trend for the capsule deficient mutant to be more susceptible to hBD-1 when compared to its parental WT strain expressing CPS. Similar findings were observed when testing for susceptibility to lysozyme (Figure 4.7). The difference in susceptibility between WT and *kpsM* for both hBD-1 and lysozyme was found not to be statistically significant. This is again mainly due to the large variation of the antimicrobial potency of hBD-1 against *C. jejuni* observed between individual experiments. The reason for this remains unclear.

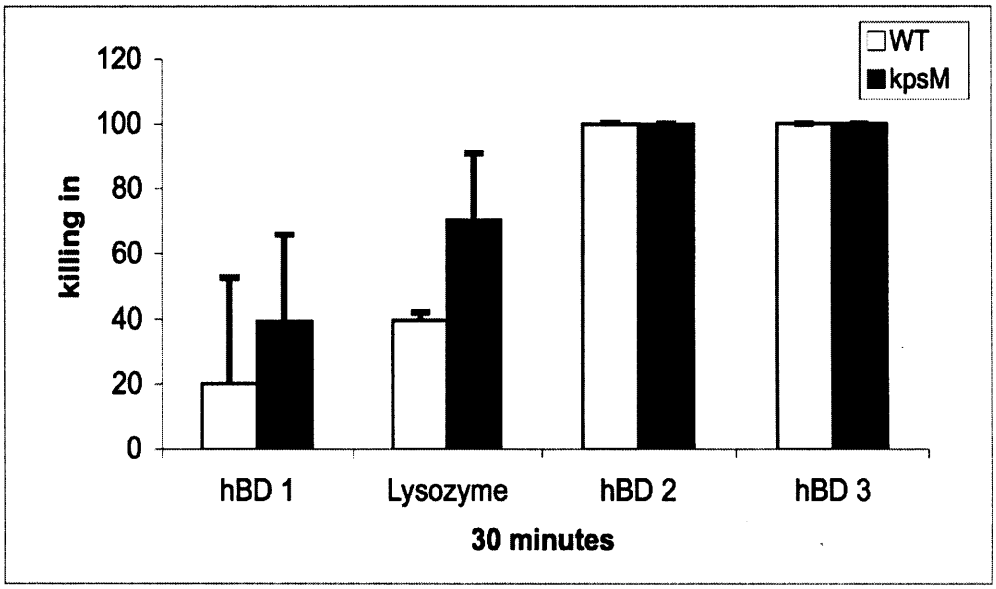



**Figure 4.7: Comparison of *C. jejuni* susceptibility to the antimicrobial action of hBD-1, -2, -3 and lysozyme, WT 11168H *versus* the capsule deficient mutant strain *kpsM*.** Bactericidal assays were performed as described above using $10^{-6}$M hBDs and $1.4x10^{-5}$M (20 μg/ml) lysozyme. Bactericidal activity of each peptide was assessed after 30min incubation. Data represents average +/-SEM of two to three independent experiments, each performed in triplicate.

### 4.3.5 Structural damage caused by hBD-3 to *C. jejuni* cell membrane

The mechanism(s) by which hBDs cause damage to bacteria remain unclear. Using SEM, the structural damage caused to *C. jejuni* 11168H by recombinant hBD-3 (the most potent bactericidal peptide against *C. jejuni*) was investigated. WT bacteria were exposed to$10^{-6}$M hBD-3 for 30min prior to preparation and examination by SEM. In parallel, viability of bacteria maintained in low salt phosphate buffer (minus hBD-3) was confirmed by comparison to *C. jejuni* incubated in Muller Hinton Broth. As shown in Figure 4.8, after 30min, control bacteria remained undamaged, retaining their spiral shape irrespective of incubation media utilised (Figure 4.8a in MH broth versus 4.8b in low salt buffer). In contrast, bacteria incubated with hBD-3 were non-viable when plated out onto solid media and showed apparent thinning out and/or pealing of the cell wall (Figure 4.9).

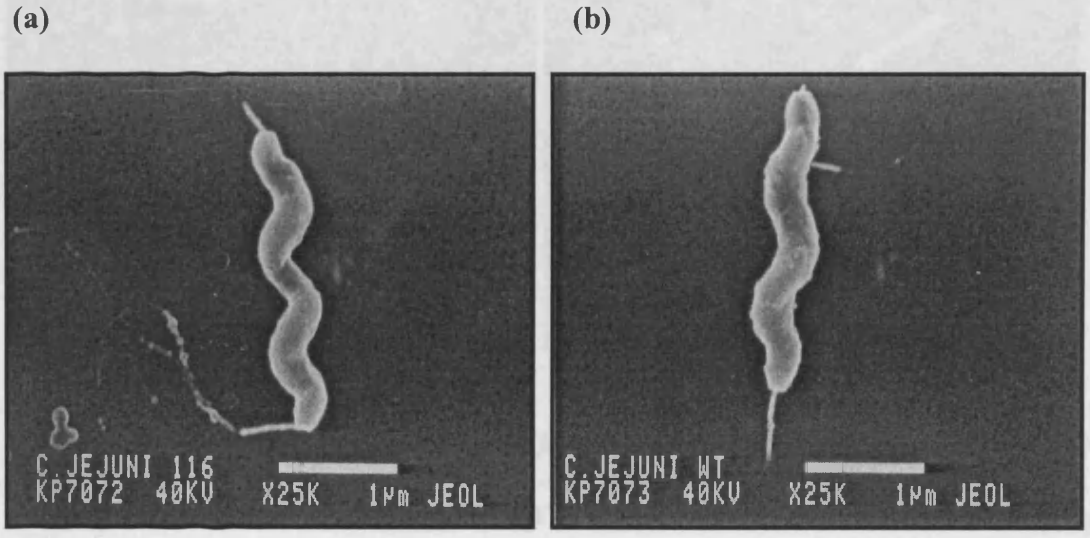



**Figure 4.8a and b: *C. jejuni* WT 11168 examined by SEM.** (a) Bacteria in Muller Hinton Broth. (b) Bacteria in low salt phosphate buffer. Bacterial spiral shape and bipolar flagella were preserved in both media.

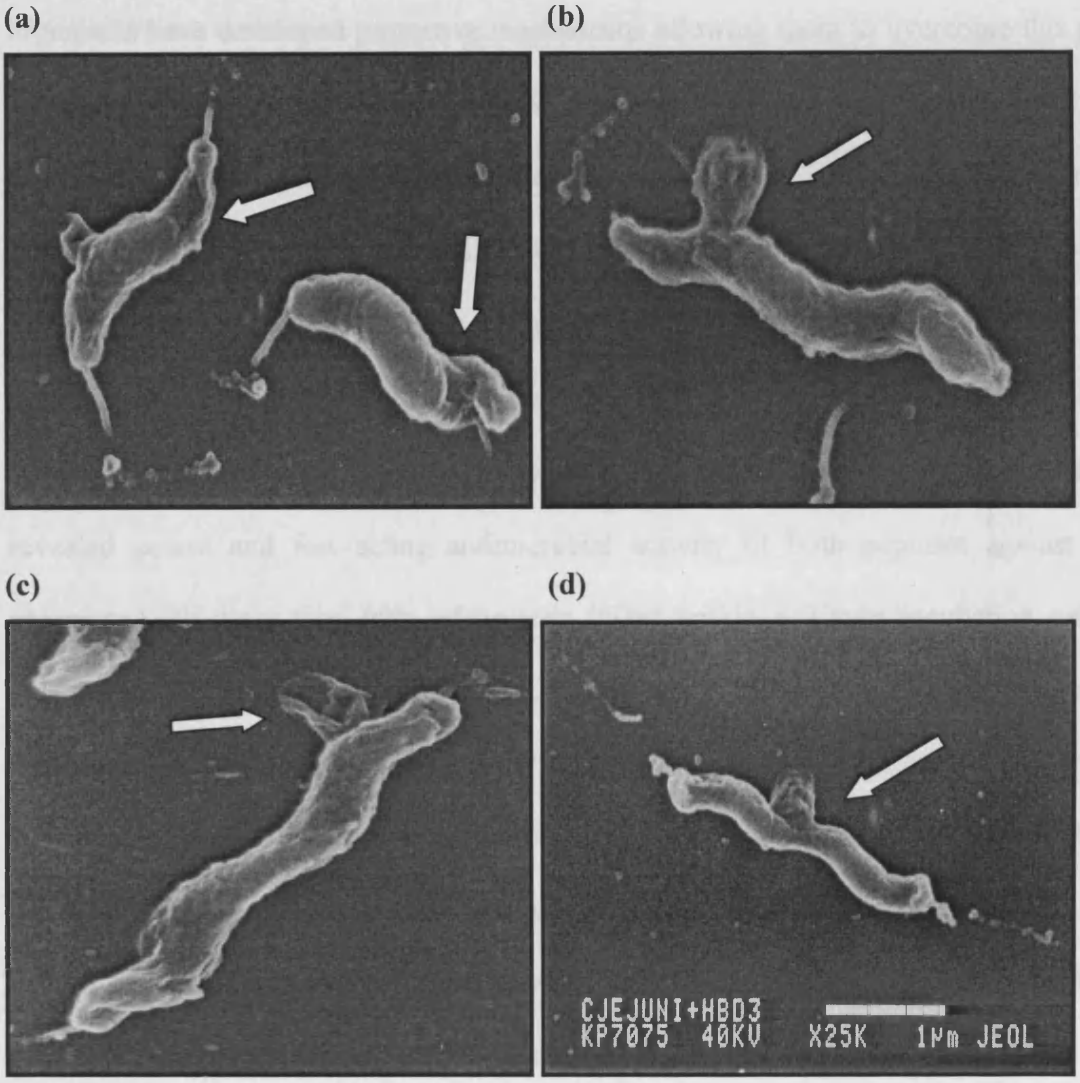



**Figure 4.9a-d: Structural damage of *C. jejuni* 11168H following exposure to hBD-3.**

WT *C. jejuni* 11168H was exposed to $10^{-6}$M hBD-3 for 30min and structural changes examined using SEM. Arrows indicate thinning of membrane (a) and pore formation (b-d). Images are representative for samples exposed to hBD-3.

## 4.4 Discussion

Defensins have been shown to exhibit antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, fungi, parasites and even enveloped viruses (Selsted and Ouellette, 2005; Jenssen *et al.*, 2006; Lehrer, 2007). However, some micro-organisms have developed protective mechanisms allowing them to overcome this part of innate host defence potentially causing disease (Peschel and Sahl, 2006). We demonstrated dynamic modulation of β-defensins by WT *C. jejuni* with induction of hBD-2 and hBD-3. In order to contribute to limiting bacterial colonisation and infection *in vivo*, β-defensins must exhibit bactericidal activity against the bacterium. We therefore investigated the susceptibility of *C. jejuni* to the antimicrobial properties of these peptides. Incubation of recombinant hBD-2 and hBD-3 with WT *C. jejuni* 11168H revealed potent and fast acting antimicrobial activity of both peptides against the organism with more than 99% of bacteria killed within a 30min incubation period (Figure 4.1). Equally potent killing of *C. jejuni* by hBD-2 and hBD-3 was observed when comparing mid-log phase bacteria to mixed culture organisms, suggesting bactericidal activity of these peptides seems to be independent of bacterial growth phase and their metabolic status (Figure 4.4). Although luminal concentrations of hBD-2 and hBD-3 in the GI tract during *C. jejuni* infections remain unknown, bactericidal dose-dependant studies shown in Figure 4.2 are compatible with those suggested for other Gram-negative bacteria, with effective killing at a micro molar range (Ghosh *et al.*, 2007). Investigating killing kinetics of hBD-3, we found evidence of lethal bacterial damage as early as 5min of exposure to the peptide, with >99.5% of bacteria rendered nonviable in this short period of time (Figure 4.3). In contrast to hBD-2 and hBD-3, the constitutively expressed hBD-1 was found not only to be significantly less potent in killing WT *C. jejuni* but also more variable, with killing ranging from 0-50% (Figure 4.1). Intriguing was the finding of higher percentage of bacterial kill by hBD-1 at 30min

post incubation compared to its activity after 60min (Figure 4.1). Furthermore, we observed a trend for mid-log phase bacteria to be more susceptible to the bactericidal activity of hBD-1 when compared to a mixed culture population, suggesting that this peptide might be more active against actively dividing bacteria (Figure 4.4). The reason for differences between inducible hBD-2/3 and constitutively expressed hBD-1 remain unclear, however, one may hypothesise that given the constitutive expression of hBD-1 its reduced bactericidal activity might be an evolutionary necessity to ensure survival of commensal bacteria. Furthermore, it may be possible that hBD-1 action is sufficient in certain circumstances to prevent bacterial colonization from proceeding for example by synergising with the effect of other constitutively expressed AMPs. Lysozyme has been shown to be differentially expressed in various parts to the upper GI tract and small intestine (Montero and Erlandsen, 1978). We confirmed constitutive expression of this AMP in our *in vitro* model (Figure 4.5) during *C. jejuni* infection and went on to investigate its bactericidal activity against the organism on its own and in combination with hBD-1. In a concentration of $1.4x10^{-5}$M (20μg/ml), lysozyme demonstrated a similar bactericidal activity against WT *C. jejuni* 11168H when compared to $10^{-6}$M hBD-1 (Figure 4.6). Combining both AMPs led to an increase in bacterial kill suggesting synergistic and/or additive effects (Figure 4.6). Taken together these findings indicate that various constitutive and/or inducible defensins may act together to provide adequate host defence.

*C. jejuni* bacterial capsule was found to play a minimal role in modulating hBD expression during infection (chapter 3). Studies by Campos *et al.* demonstrated that CPS of *K. pneumoniae* mediated protection against AMPs (Campos *et al.*, 2004), we hypothesised similar findings for *C. jejuni.* Surprisingly, WT *C. jejuni* 11168H was found to be equally susceptible to the antimicrobial action of hBD-2 and hBD-3 when compared to the capsule deficient mutant strain *kpsM* suggesting a minor role for this

bacterial component in mediating protection (Figure 4.7). We consistently observed greater efficiency of hBD-1 in killing the *kpsM* mutant when compared to the parental WT strain (Figure 4.7). Similar observations were also noted for lysozyme, which was slightly more effective against the isogenic CPS deficient mutant strain (Figure 4.7). Despite the lack of statistical significance, these observations point towards a potential role of *C. jejuni* CPS in providing protection against constitutively expressed AMPs, a possible advantage in early bacterial-epithelial contact, aiding the bacterium to adhere and invade the intestinal epithelium. There is some evidence that *C. jejuni* down-regulates CPS as the bacteria adheres to and invades IECs (N. Dorrell and P. H. Everest, unpublished data). This would not only support the lack of protection against AMPs but also explain our findings suggesting that *C. jejuni* capsule may not be involved in modulating intestinal epithelial innate immune responses.

To date the mechanism(s) by which defensins mediate their antimicrobial activity remain unclear. However, evidence is accumulating that following the initial contact of the defensin peptide with the target cell, membrane permeabilization can occur (Sahl *et al.*, 2005; Jenssen *et al.*, 2006). Scanning electron microscopy allowed visualisation of the structural damages to WT *C. jejuni* 11168H caused by hBD-3. Following 30min incubation we observed peeling of bacterial membrane accompanied by disruption of cell wall leading to spillage of cellular contents (Figure 4.9a-d). Importantly, these changes were absent in bacteria resuspended in buffer alone suggesting these effects are most likely specific to defensin action.

In conclusion, data presented in this chapter lends further support to our hypothesis that β-defensins play an important role in *C. jejuni* disease pathogenesis. Specifically, with expression of hBD-2 and hBD-3 being induced by *C. jejuni* combined with their potent and fast acting antimicrobial activity against the bacterium, these AMPs might

contribute to the self-limiting nature of disease *via* enhanced clearance in the healthy host.

# CHAPTER 5

# The role of intestinal epithelial Nucleotide Oligomerisation Domain 1 (NOD1) in mediating host innate response to *C. jejuni*

## 5.1 Introduction

Prior to the induction of innate defence, host cells are required to sense the presence of potential pathogens. In the GI tract the constant presence of the diverse enteric microflora adds further complexity to this task. The intestinal epithelium as front line of defence faces the challenge to discriminate between pathogens and harmless commensal bacteria. Mechanisms leading to the observed tolerance of the intestinal epithelium towards luminal commensals and their products (Cario and Podolsky, 2005) include the reduced surface expression of TLRs (i.e. TLR4) (Cario and Podolsky, 2000; Abreu *et al.*, 2001; Naik *et al.*, 2001) and tight control of their signalling such as expression of the TLR-signaling suppressor Tollip (Melmed *et al.*, 2003). In contrast, expression of cytoplasmic PRRs (i.e. of the NOD family) allows recognition of invaded bacteria and presence of cytoplasmic bacterial products (Kufer *et al.*, 2006).

As a well recognised early event in host *C. jejuni* interaction, several studies have investigated mechanisms involved in *C. jejuni* mediated IEC IL-8 production (Hickey *et al.*, 1999; Hickey *et al.*, 2000; Watson and Galan, 2005; Johanesen and Dwinell, 2006). Both bacterial structural [e.g. cytolethal distending toxin (CDT)] components and active processes such as adhesion/invasion and *de novo* protein synthesis have been implicated in eliciting epithelial innate immune responses (Hickey *et al.*, 2000; Mellits *et al.*, 2002; Watson and Galan, 2005). However, despite these advances in our understanding of the role and contribution of bacterial factors implicated in *C. jejuni*-mediated epithelial responses, at present our knowledge remains rudimentary as to how the host senses the presence of the bacterium and initiates an effective immune response.

Recent evidence suggesting that *C. jejuni* flagellin is a poor ligand for IEC TLR5 (Watson and Galan, 2005; Johanesen and Dwinell, 2006), combined with the observed association of bacterial invasion and innate immune activation (Hickey *et al.*, 1999), led us to hypothesise that cytoplasmic NOD proteins might be involved in host sensing of

*C. jejuni* (Inohara *et al.*, 2005). NOD1 (encoded by the caspase-recruitment domain 4 gene; *CARD4*) and NOD2 (encoded by *CARD15*) recognize components of peptidoglycans (PGNs), ubiquitous constituents of bacterial cell walls (Inohara and Nunez, 2003; Inohara *et al.*, 2005; Franchi *et al.*, 2006). The minimal PGN structure that acts as a specific ligand for NOD1/*CARD4* is γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP), a signature motif found in most Gram-negative and some Gram-positive bacterial PGNs (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003b). In contrast, NOD2/*CARD15* is a more global bacterial sensor as it interacts with N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP), a motif common to both Gram-negative and Gram-positive bacterial PGNs (Girardin *et al.*, 2003a; Inohara and Nunez, 2003).

In the following chapter we investigated the potential role of NOD1 and NOD2 as cytoplasmic PRRs involved in initiating host IEC innate immunity in response to *C. jejuni*.

## 5.2 Methods

### 5.2.1 Bacterial strains and culture conditions

The *C. jejuni* wild-type strains used were as described in chapter 2. WT encapsulated *Streptococcus pneumoniae (S. pneumoniae,* serotype F19) was kindly provided by Dr. Helen Baxendale (Department of Infectious Diseases and Microbiology, Institute of Child Health, London UK). *S. pneumoniae* were grown on Columbia agar plates supplemented with 7% v/v defibrinated horse blood at 37°C with 5% $CO_2$ over 24h. Single colonies were resuspended in Todd-Hewitt broth (Sigma-Aldrich, Gillingham, UK) supplemented with 0.5% yeast extract and incubated at 37 °C for 3–4h (100 rpm) to reach mid-logarithmic phase ($A_{600}$ = 0.2–0.4). Bacteria were harvested by centrifugation and resuspended in tissue culture medium (DMEM minus antibiotics) to a final concentration of 2 x $10^8$ CFU/ml.

### 5.2.2 Localisation of *C. jejuni* in Caco-2 co-cultures by confocal microscopy

#### 5.2.2.1 Fluorescent labeling and PFA treatment of *C. jejuni* 81-176

Prior to bacterial co-culture, fluorescent labeling of WT *C. jejuni* 81-176 (approximately 1 x $10^8$ CFU/ml) was performed by incubating bacteria in PBS containing 0.1mg/ml Fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Gillingham, UK) for 15min at RT. Bacteria were washed twice in PBS to remove unbound FITC and resuspended in antibiotic free DMEM medium supplemented with 1% FCS. Fixation of one proportion of FITC-labeled *C. jejuni* 81-176 was achieved by incubation in paraformaldehyde (PFA 4%) for 15min at RT, followed by 3 washes in tissue-culture media to remove residual traces of PFA. 100% inactivation by PFA treatment was confirmed by plating out bacterial suspension on blood agar plates followed by viable counting after 2 to 3 days.

#### 5.2.2.2 Bacterial co-culture with FITC-labeled *C. jejuni* (live *versus* PFA fixed)

Caco-2 cells were grown on cover slips in 24 well plates over 7 days to reach complete confluency. Co-culture of Caco-2 cells with live or PFA fixed FITC-labeled *C. jejuni* (MOI of 100) was carried out in DMEM for 5h to allow for adhesion and invasion to occur. Following incubation time, cells were washed twice with PBS and fixed in 4% PFA for 20min at RT.

#### 5.2.2.3 Immuno-labeling of extracellular *C. jejuni* post co-culture

Following 2 washes in PBS to remove residual PFA, non-specific binding was blocked by incubating cells with 1% BSA (in PBS) for 45 min. Extracellular bacteria were labelled with primary unconjugated goat anti-*C. jejuni* antibodies (KPL, Maryland, USA) at a concentration of 5µg/ml for 60min at RT, followed by incubation with Alexa Fluor 568-conjugated rabbit anti-goat IgG (Molecular Probes, Eugene, Oregon, USA) for 30min. Bacteria and cell nuclei were counterstained with TO-PRO-3 (Molecular Probes) for 30min. After each step, cells were washed 3 times with PBS. Co-cultures

were visualised with a Radiance 2100 confocal laser scanning microscope equipped with an Argon-Krypton laser and a red diode (Bio-Rad, UK). As intracellular bacteria were protected from antibody labelling, they appear as green (in merged image). In contrast, extracellularly located *C. jejuni* appear yellow due to double labelling in merged image.

### 5.2.3 Small interfering (si) RNA experiments

Pre-designed siRNA sequences targeting NOD1 (Sequence 1: sense GGC CAA AGU CUA UGA AGA Utt, Sequence 2: sense GGG UGA GAC CAU CUU CAU Ctt) and a non-targeting negative (siNEG) control sequence (catalogue number AM 4611) were all purchased from Ambion (Huntingdon, Cambridge UK). Caco-2 cells were reverse transfected with siRNA using siPORT NeoFX Transfection Reagent (Ambion). This method allowed transfection to occur during the initial phase of cell adherence.

siRNA was prepared in Opti-MEM serum-free medium (Invitrogen) by mixing 0.7 or 0.8µl (per well) of the transfection reagent with 10 or 20nM siRNA respectively at RT for 10min. Caco-2 cells ($8x10^3$cells/well) were then transferred to a 96-well plate containing siRNA-transfection reagent complexes and mixed by gentle flicking of the plate. Media containing transfection reagent was replaced after 20h by complete media (allowing cell recovery) and incubation continued for an additional 28h prior to bacterial infection. Gene knock-down was confirmed by RT-PCR. For luciferase reporter gene assays, a second transfection step was performed 30h post reverse transfection of siRNA.

### 5.2.4 Transfection of Caco-2 cells with NOD2 overexpression plasmid

NOD2 overexpression plasmid was kindly provided by Professor G. Núñez (Department of Pathology and Comprehensive Cancer Centre, University of Michigan Medical School, Ann Arbor, Michigan, USA). 100ng NOD2 plasmid was transfected into Caco-2 ($8x10^3$) cells using FuGene reagent (Roche, Lewes, UK; chapter 2). For luciferase reporter gene assays, NOD2 plasmid was co-transfected with IL-8 or hBD2 promoter constructs. NOD2 expression was confirmed by RT-PCR and bacterial co-culture studies performed 48h post-transfection.

### 5.2.5 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from the blood of healthy donors using Lymphoprep Ficoll centrifugation gradient (Sigma, Poole, UK). Approximately 20mls of blood was diluted with an equal volume of RPMI 1640 + glutamine (Invitrogen, Paisley, UK). The diluted blood was slowly layered onto 20mls of Ficoll lymphoprep. Resulting solution was centrifuged (1800rpm, 30min, no breaks; Rotina 46R centrifuge, Wolf Laboratories, UK) and PBMCs removed from the buffy coat layer (interface between the serparating gel and plasma layer) using a sterile Pasteur pipette. Approximately 15mls of the obtained cell suspension was re-centrifuged at 1600rpm for 10min to remove traces of lymphoprep. The resultant pellet was subjected to RNA extraction and RT-PCR.

### 5.2.6 *C. jejuni* invasion assay

Initially, a standard gentamicin protection assay was optimized for *C. jejuni* in Caco-2 cells. This included adjustment of incubation time sufficient to allow for adhesion and invasion, concentration of gentamicin and exposure time to ensure sufficient kill of extracellular bacteria. Following optimization, the assay was adjusted to use in transfected cells as follows: Caco-2 cells were plated in a 96-well plate and reverse

transfected with NOD1 siRNA or over-expressing NOD2 plasmid. After 48h approximately $10^7$ CFU/ml of *C. jejuni* 81-176 cells were added to each well. Following a 20h infection period, cells were incubated in media containing gentamicin (150μg/ml) for 60min. This procedure ensured complete kill of any adherent extracellular bacteria. Cells were washed 3 times with PBS prior to lysis with 0.2% v/v Triton X-100 (in PBS) for 15min at RT. Serial dilutions were plated onto blood agar plates and percentage of invasion calculated by counting viable CFU in the lysate compared to control untreated Caco-2 cells.

## 5.3 Results

### 5.3.1 Intracellular *C. jejuni* bacterial component(s) play an important role in eliciting IEC innate immunity

Prior to investigating the role of cytoplasmic NOD proteins as potential PRR(s) for *C. jejuni*, we wished to establish a link between epithelial responses and bacterial localisation in our co-culture model system. For this purpose, we compared epithelial responses of live *versus* PFA-fixed *C. jejuni.* PFA treatment allows maintenance of the structural integrity of the bacterial cells (albeit with reduced ability for conformational changes) while rendering the bacteria non-viable and unable to execute and participate in active, energy requiring processes. We observed potent induction of epithelial IL-8 and hBD-2 gene expression in the presence of infection with live *C. jejuni* 11168H and 81-176 cells, with the response markedly reduced on exposure to PFA-fixed cells (Figure 5.1a and b). Potency of infection with live bacteria was also confirmed at the protein level. In comparison PFA-fixed bacteria showed a significant reduction in their ability to induce IL-8 protein (Figure 5.2). Bacterial localisation was investigated 5h post-infection. *C. jejuni* was detected both intracellularly (green) and extracellularly (yellow; merged image) following infection with live 81-176 strain (Figure 5.3; merged

upper-panel). In contrast, PFA-fixed bacteria showed mainly extracellular localisation (Figure 5.3; merged lower panel)

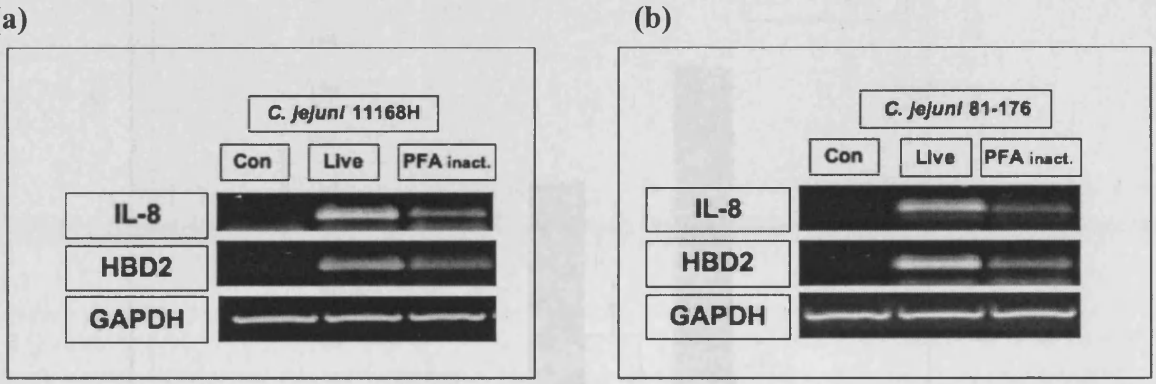



**Figure 5.1a and b: Induction of IL-8 and hBD-2 by PFA fixed *versus* live *C. jejuni*.**

Co-culture experiments were performed in Caco-2 cells with live and PFA fixed WT *C. jejuni* strains 11168H (a) and 81-176 (b) at an MOI of 100. 8h post-infection, IL-8 and hBD-2 gene expression was analyzed by RT-PCR. Experiments were performed at least three times, a representative gel is shown.

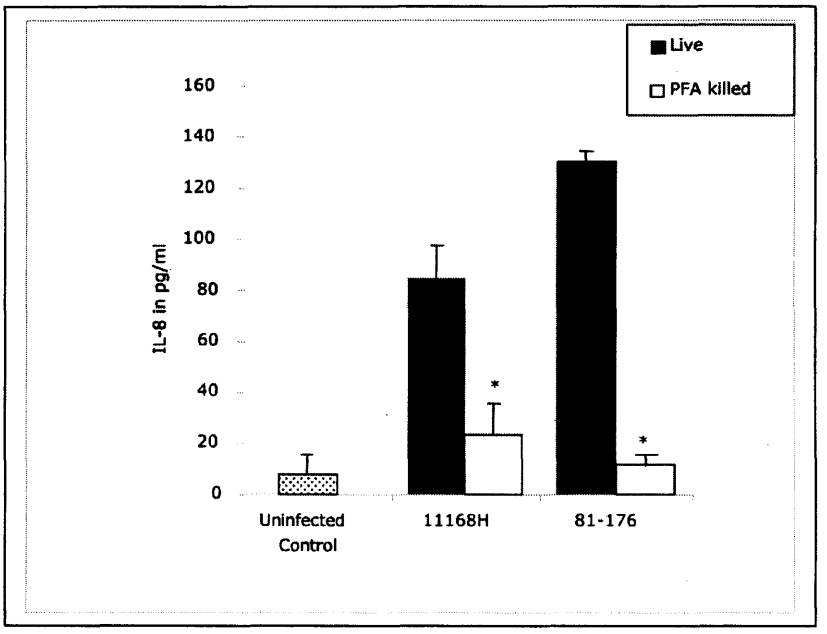



**Figure 5.2: Induction of IL-8 protein levels by live *versus* PFA fixed *C. jejuni.*** Caco-2 cells were infected with live and PFA fixed *C. jejuni* WT strain 11168H and 81-176. 24h post infection, IL-8 levels were analysed in culture supernatants by ELISA. Error bars indicate +/-SEM obtained from a minimum of two independent experiments performed in duplicates. *, $P < 0.05$; live *versus* PFA-fixed *C. jejuni* cells.

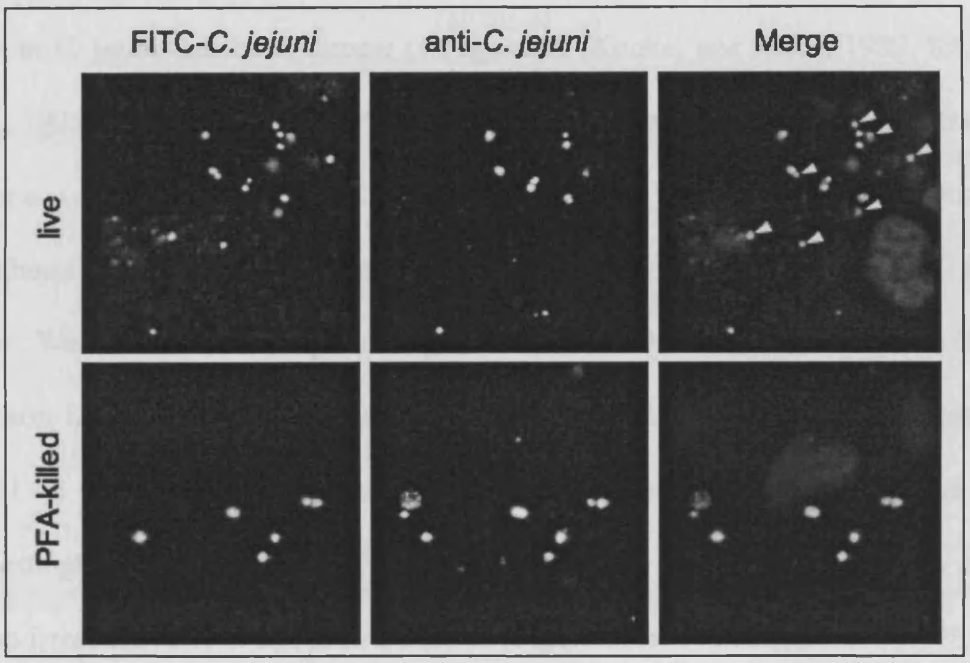
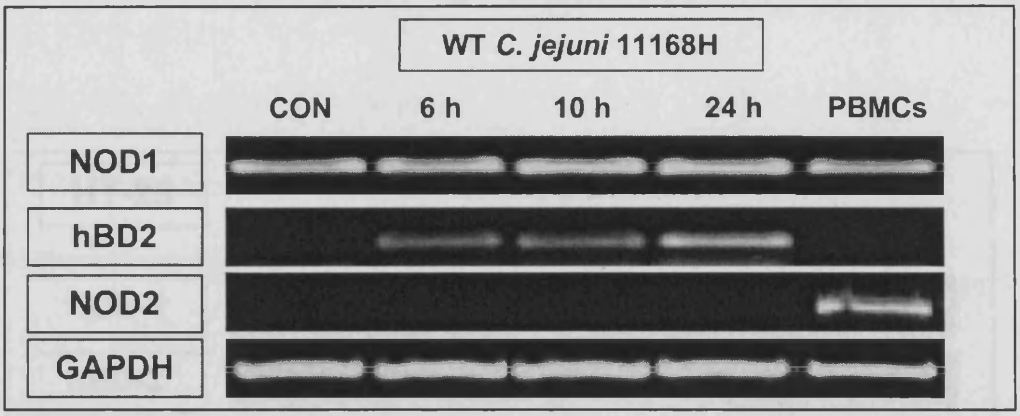



**Figure 5.3: Cellular localization of live and PFA fixed *C. jejuni* cells 5h post-infection.** Caco-2 cells were grown on cover-slips and co-cultured with live or PFA-fixed FITC-labelled *C. jejuni* 81-176 (green) for 5h. To distinguish between intra- and extracellular bacterial localization, non-permeabilised cells were stained with anti-*C. jejuni* antibodies followed by Alexa Fluor 568 conjugated rabbit anti-goat IgG. The latter treatment allowed for additional staining of extracellular FITC labeled bacteria leading to a yellow appearance (merging of green FITC and red Alexa Fluor-568). In contrast, intracellular bacteria remained green. Nuclei were counterstained with TO-PRO-3 (blue in merged image). Intracellular bacterial localization is highlighted (arrowheads). A representative image from 2 experiments (performed in triplicates) is shown.

### 5.3.2 Intestinal epithelial NOD1 and NOD2 gene expression during *C. jejuni* infection

Bacterial adherence to and subsequent invasion of the IEC is a well established early event in *C. jejuni*-mediated disease pathogenesis (Konkel and Joens, 1989; Szymanski *et al.*, 1995; Monteville *et al.*, 2003). The causal association of presence of intracellular bacteria with potent IEC responses (Figure 5.1-5.3) added weight to our initial hypothesis that epithelial, cytoplasmic NOD protein(s) may be potential PRR(s) for *C. jejuni*. We therefore investigated expression of NOD1 and NOD2 during *C. jejuni* infection in our tissue culture model. As shown in Figure 5.4a and b, expression of NOD1 in Caco-2 cells was found to be constitutive (Figure 5.4a-b; upper panel). Interestingly, no modulation of NOD1 expression was noted during the 24h infection period irrespective of bacterial strain tested (Figure 5.4a: 11168H, 5.4b: 81-176). These findings were confirmed using HT-29 cell line (Figure 5.5a and b). Evidence for infectivity was sought in parallel. This was investigated by following the expression of hBD-2; whose expression is known to be dependent on NOD1 engagement (Boughan *et al.*, 2006). No hBD-2 expression was noted in control; uninfected Caco-2 or HT-29 cells (Figure 5.4 and 5.5; second panel). In response to infection of both cell-lines with two different wild-type strains (11168H and 81-176), we observed induction of hBD-2; with initial expression noted as early as 6h post-infection (Figure 5.4 and 5.5; second panel). To delineate a role for NOD2 as a potential PRR for *C. jejuni*, we followed the expression of this molecule during infection. NOD2 expression was undetectable in either uninfected or *C. jejuni*-infected Caco-2 or HT-29 cell-lines throughout the 24h course of infection (Figure 5.4 and 5.5;third panel). Macrophages in PBMCs are known to express NOD2 and were therefore included in each experiment as a positive control.

(a)




(b)

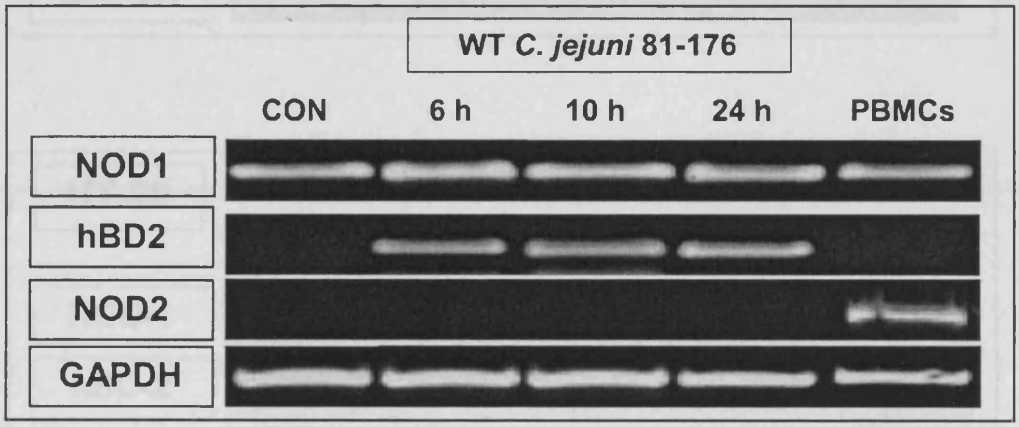
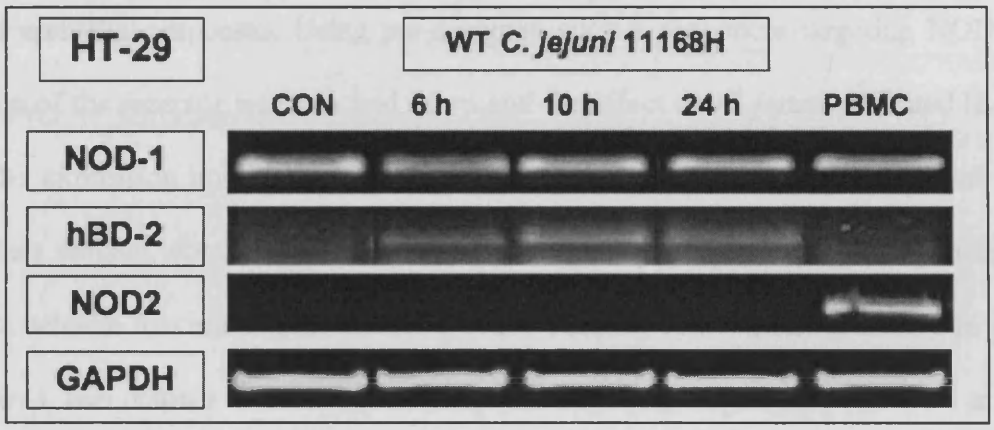



**Figure 5.4a and b: Expression of NOD1 and NOD2 in Caco-2 cells during *C. jejuni* infection.** Caco-2 cells were co-cultured with *C. jejuni* wild-type 11168H (a) or 81-176 (b) strains at an MOI of 100 for up to 24h. Time-dependant gene expression of NOD1 (upper panel) and NOD2 (third panel) was followed by RT-PCR. In parallel, evidence for infectivity was confirmed by analysing induction of hBD-2 expression (second panel). Peripheral blood mononuclear cells (PBMCs) served as positive control for NOD2. Experiments were done at least 3 times, a representative gel is shown.

(a)




(b)

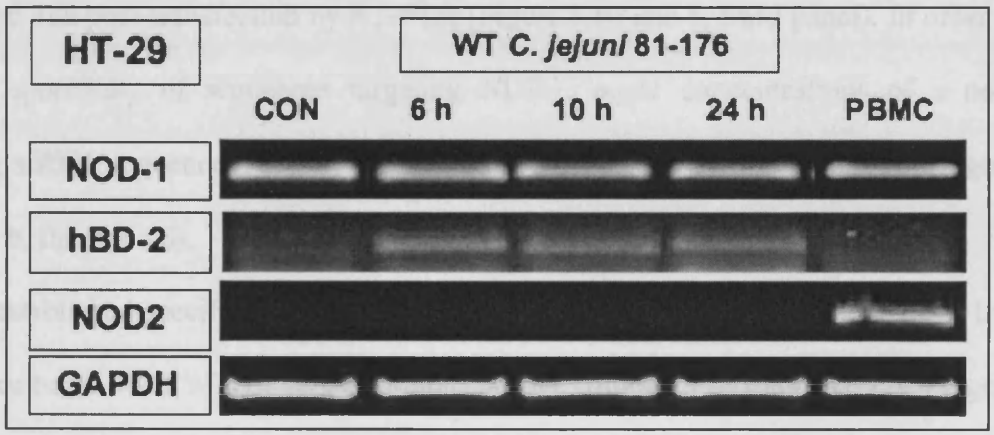


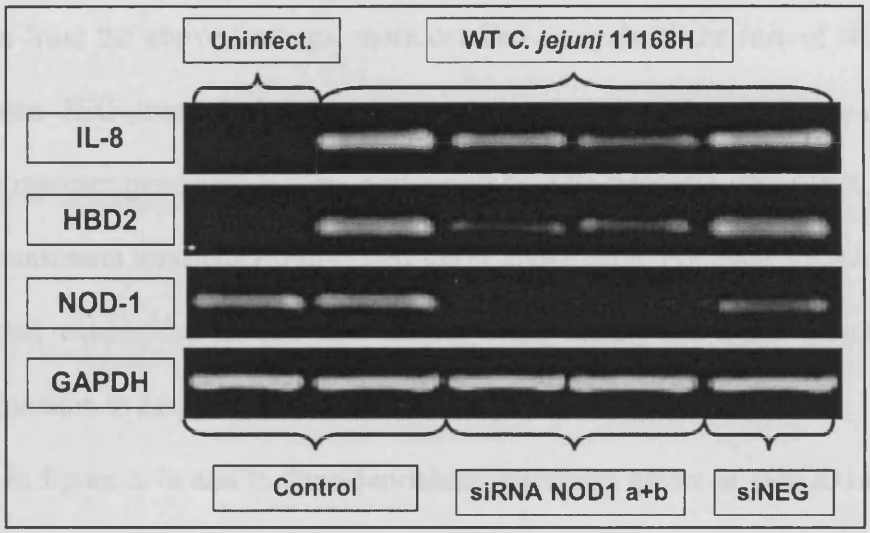

**Figure 5.5a and b: Expression of NOD1 and NOD2 in HT-29 cell line during *C. jejuni* infection.** HT-29 cell line was infected with WT *C. jejuni* 11168H (a) and 81-176 (b) at an MOI of 100 over 24h. Expression of NOD1 (upper panel) and NOD2 (third panel) was analyzed by RT-PCR. Infectivity was confirmed by demonstrating induction of hBD-2 (second panel). A representative gel of 3 independent experiments is shown.

### 5.3.3 The presence of NOD1 small interfering (si) RNA inhibits IL-8 and hBD-2 gene expression in response to *C. jejuni* infection

We next wished to establish if intestinal epithelial NOD1 was involved in *C. jejuni*-mediated epithelial responses. Using pre-designed siRNA sequences targeting NOD1, expression of the receptor was knocked down and the effect on *C. jejuni* mediated IL-8 and hBD-2 expression investigated. During initial optimization experiments, amount of transfection reagent, concentration of siRNA and incubation period were established aiming to achieve maximum gene silencing while keeping cell toxicity minimal. In all experiments, two distinct siRNA sequences specifically targeting NOD1 (siNOD1 a & b) were transiently transfected into Caco-2 cell line and NOD1 gene knock down confirmed 48h post-transfection by RT-PCR (Figure 5.6a and b, third panel). In order to confirm specificity of sequences targeting NOD1, equal concentrations of a non-targeting siRNA sequence (siNEG) was routinely included as a negative control (Figure 5.6a and b, third panel).

Having established specific knock down of NOD1 receptor, transfected Caco-2 cell line was co-cultured with WT *C. jejuni* strain 11168H (Figure 5.6a) and 81-176 (Figure 5.6b) strains for 8h. As shown in Figure 5.6a and b, IL-8 (top panel) and hBD-2 (second panel) gene expression was significantly inhibited following transfection with siNOD1 (a & b), while siNEG did not exhibit any significant effect.

(a)




(b)

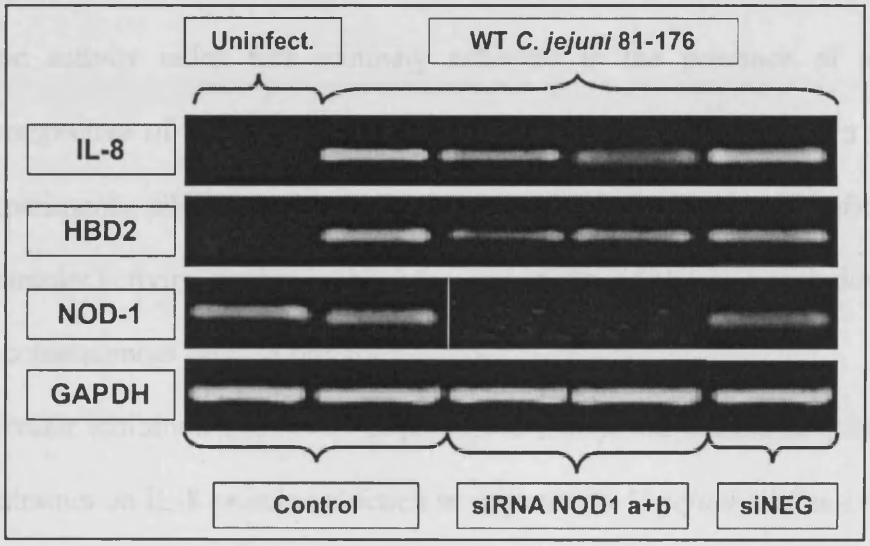



**Figure 5.6a and b: Effect of siNOD1 on *C. jejuni* mediated induction of IL-8 and hBD-2 gene expression.** Caco-2 cell line was reverse transfected with two distinct siRNA sequences targeting NOD1 (siNOD1 a and b) and a non-targeting (siNEG) sequence. 48h post transfection, cells were co-cultured with *C. jejuni* WT strains 11168H (a) and 81-176 (b) for 8h. Expression of IL-8, hBD-2, NOD1 and GAPDH was followed by RT-PCR. A representative gel of three independent experiments is shown.

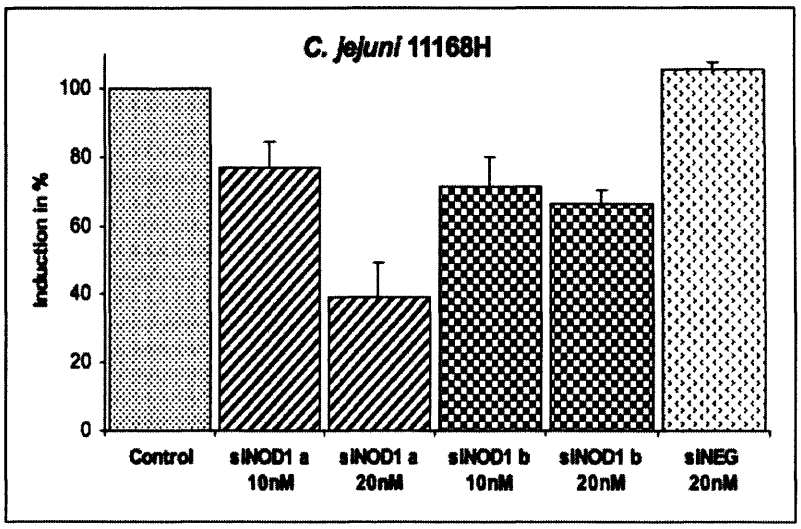
### 5.3.4 Reduced NOD1 expression inhibits *C. jejuni* mediated IL-8 promoter activation and protein secretion

Leading on from the above findings, more detailed analysis of the role of NOD1 in *C. jejuni* driven IEC transcriptional and translational innate immunity was sought. Luciferase reporter gene assays were performed in cells treated with siRNA, and IL-8 promoter constructs transiently transfected into Caco-2 cells. For each siRNA sequence two different concentrations (10 and 20nM) were tested and their effect on IL-8 promoter function in response to *C. jejuni* investigated.

As shown in figure 5.7a and b, dose-dependent inhibitory effect of siNOD1a and b on *C. jejuni*-mediated IL-8 promoter function was observed. 30-50% inhibition of IL-8 transcription activity index was routinely achieved in the presence of transfected siNOD1 irrespective of the bacterial strain or sequence tested (Figure 5.7a and b). In parallel experiments, siNEG sequence (20nM) was found to have no significant effect on IL-8 promoter activity, further highlighting specificity of NOD1 knock down and its biological consequences on IL-8 promoter activity.

Once the greater inhibition of siNOD1 sequences at 20nM was established, the effect of this concentration on IL-8 protein induction in response to *C. jejuni* strains 11168H and 81-187 was tested. Following transfection of siRNA sequences, Caco-2 cells were infected with WT *C. jejuni* over 24h and IL-8 concentration measured by ELISA in culture supernatants. As shown in Figure 5.8, induction of IL-8 protein in response to infection with *C. jejuni* 11168H and 81-176 strains was significantly inhibited in the presence of both siNOD1 a and b sequences with siNEG exhibiting no significant effect.

(a)




(b)

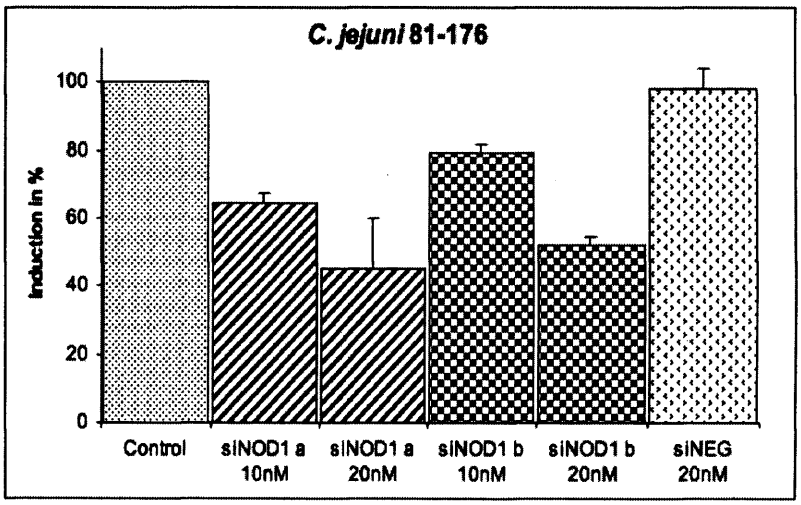



**Figure 5.7a and b: Dose-dependent effect of siNOD1a and b on *C. jejuni*-mediated IL-8 promoter function.** IL-8 promoter constructs were transiently transfected in Caco-2 cell line following treatment with siNOD1a and b (10 and 20nM) and siNEG (20nM). 48h post transfection, cells were co-cultured with WT *C. jejuni* 11168H (a) and 81-176 (b) for 8h and promoter-luciferase activity assessed. IL-8 promoter activity in uninfected and *C. jejuni* infected Caco-2 cell line was included, the latter representing 100% induction. Data represent the average +/-SEM three independent experiments performed in triplicates.

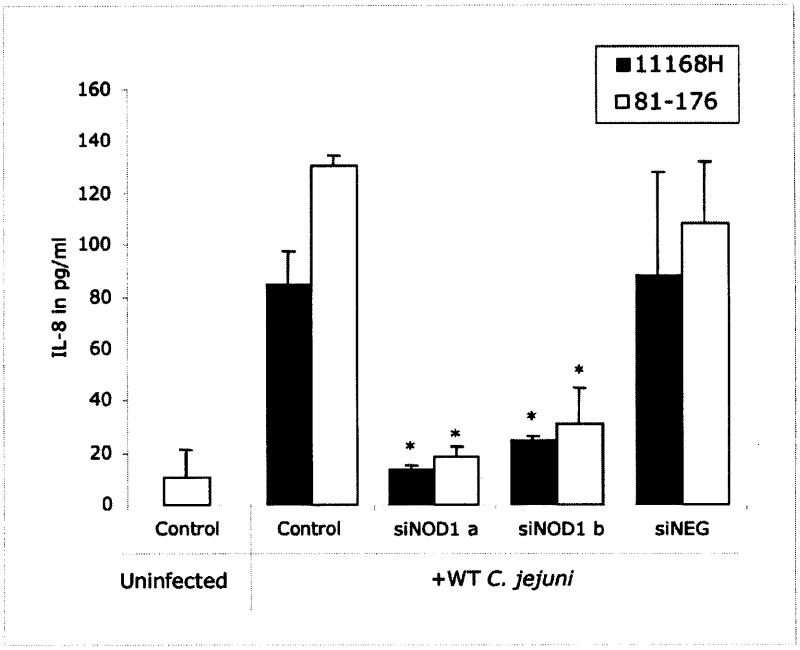



**Figure 5.8: Effect of siNOD1 on *C. jejuni*-mediated IL-8 protein production.** Caco-2 cells were treated with siRNA targeting NOD1 (siNOD1a and b) and non targeting negative control (siNEG). 48h post treatment, cells were infected with *C. jejuni* WT strains 11168H and 81-176 for 24h and IL-8 measured in cell supernatants. Data presented are average +/- SEM of two independent experiments performed in duplicates. *, $P<0.05$ *versus* IL-8 induction in untreated and non targeting siRNA treated Caco-2 cells.

### 5.3.5 Silencing of NOD1 expression inhibits *C. jejuni* mediated hBD-2 promoter activation

The role of NOD1 for *C. jejuni* mediated hBD-2 promoter activity was also investigated. As for IL-8, hBD-2 promoter constructs were transiently transfected in siRNA treated Caco-2 cells (20nM) followed by co-culture with WT *C. jejuni* 11168H (Figure 5.9a) and 81-176 (Figure 5.9b). 50-60% reduction in *C. jejuni*-mediated hBD-2 promoter function in the presence of siNOD1 a and b was observed. Importantly, the presence of siNEG had minimal effect on hBD-2 promoter function (Figure 5.9).

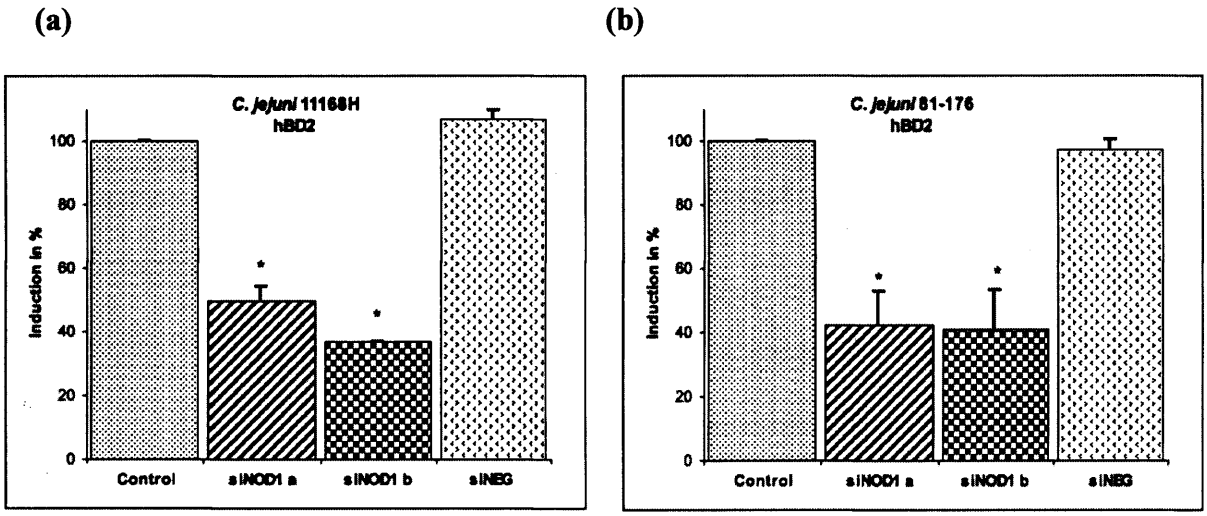



**Figure 5.9a and b: Effect of siNOD1 on *C. jejuni*-mediated hBD-2 promoter function.** HBD-2 promoter constructs were transiently transfected in Caco-2 cells following treatment with siNOD1a+b and siNEG (20nM). 48h post transfection, cells were co-cultured with WT *C. jejuni* 11168H (a) and 81-176 (b) for 8h and promoter-luciferase activity assessed. HBD-2 promoter activity in untreated, *C. jejuni* infected Caco-2 cells were included as controls, the latter representing 100% induction. Data represent the average +/- SEM of three independant experiments performed in triplicates. *, $P<0.05$ *versus* hBD-2 induction in untreated and non targeting siRNA treated Caco-2 cells.

### 5.3.6 NOD2 plays a minimal role in intestinal epithelial immune defence against *C. jejuni*

Experiments described above showed that NOD2 gene expression was not seen in resting or *C. jejuni* stimulated epithelial cells (Figure 5.4 and 5.5). However, during an *in vivo* infection, intestinal epithelial homeostasis is likely to be influenced indirectly by neighbouring inflammatory immune cells and their secretory products such as cytokines, molecules known to modulate NOD2 expression (Gutierrez *et al.*, 2002; Rosenstiel *et al.*, 2003). To establish if epithelial NOD2 (when expressed under inflammatory conditions) may also act as a potential PRR for *C. jejuni,* we transiently transfected Caco-2 cells with a plasmid over-expressing NOD2. NOD2 gene expression 48h post-transfection was confirmed by RT-PCR (Figure 5.10a and b; third panel). Control and NOD2 transfected Caco-2 cells were exposed to *C. jejuni* wild-type strains 11168H (Figure 5.10a) and 81-176 (Figure 5.10b). Infection with either strain resulted in no enhanced IL-8 or hBD-2 gene expression (Figure 5.9a and b). These findings were confirmed by using promoter constructs for both IL-8 and hBD-2. As shown in Figure 5.10c and d, no increase in *C. jejuni*-mediated IL-8 and hBD-2 promoter function was noted in the presence of NOD2 when compared to control cells lacking NOD2 (Figure 5.10c and d).

*Streptococcus pneumoniae* (a causative agent for bacterial meningitis) transiently invades epithelial cells and utilises NOD2 as a PRR (Opitz *et al.*, 2004). To confirm that the transfected NOD2 gene in our cell culture system was biologically active, we included S. *pneumoniae* infection as a control. As noted for gene expression, the presence of NOD2 had no effect on the levels of IL-8 protein production in response to *C. jejuni* strains 11168H or 81-176 (Figure 5.11). In contrast, a significant increase in IL-8 protein levels was observed in NOD2-transfected Caco-2 cells in response to S. *pneumoniae* compared to infection in control, untransfected cells.

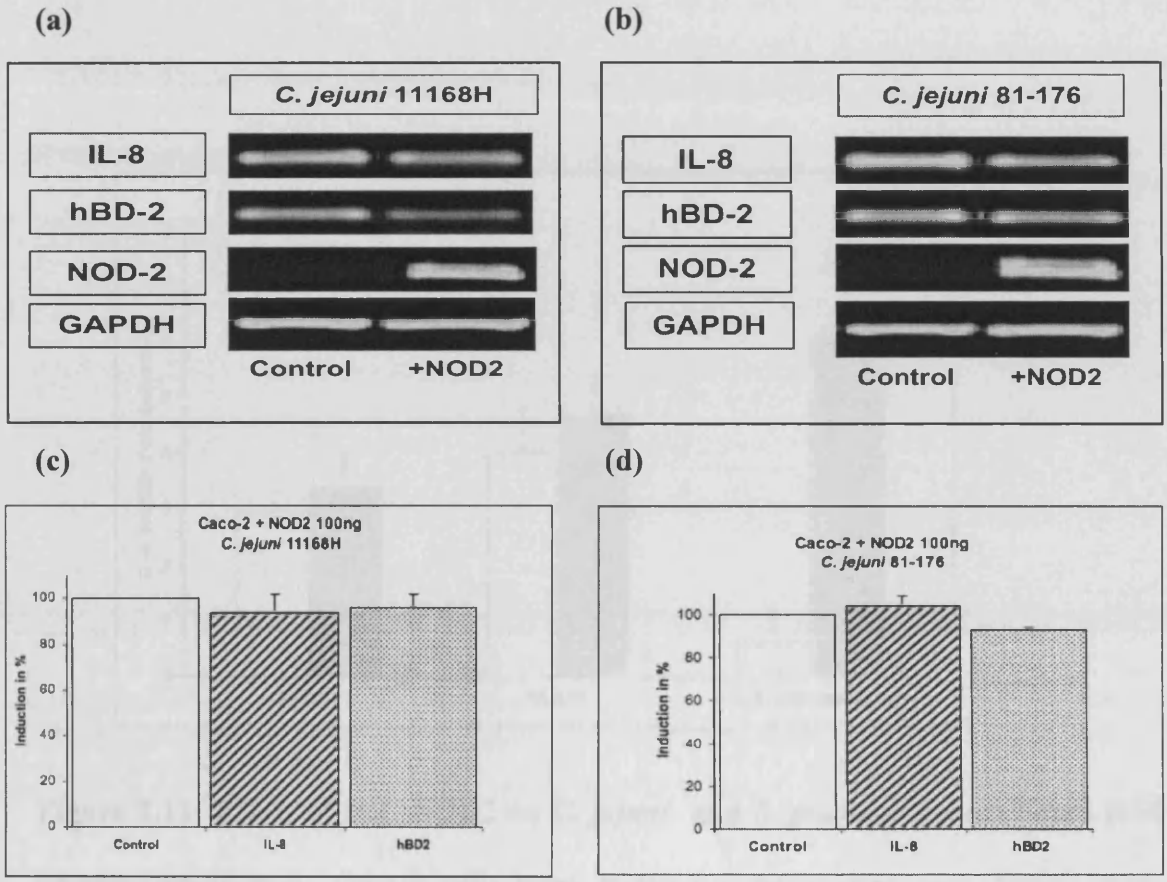



**Figure 5.10a-d: Effect of IEC NOD2 in *C. jejuni*-mediated IL-8 and hBD-2 induction.** Caco-2 cells were transiently transfected with NOD2 over-expressing plasmid for 48h followed by infection with *C. jejuni* WT 11168H (a and c) or 81-176 (b and d) strains for 8h. IL-8 and hBD2 gene expression was analyzed by RT-PCR and a representative gel of 3 independent experiments is shown (a and b). Transcriptional activity was also quantified by promoter luciferase reporter assay (c and d). Data represents +/-SEM of three experiments performed in triplicates.

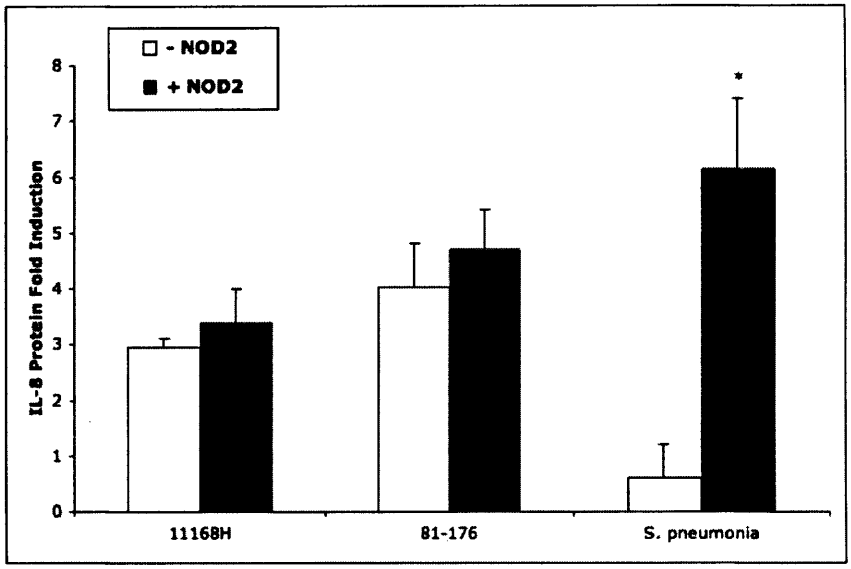



**Figure 5.11: Effect of IEC NOD2 on *C. jejuni*- and *S. pneumoniae*-mediated IL-8 protein production.** Caco-2 cells were transiently transfected with NOD2 over-expressing plasmid for 48h followed by infection with *C. jejuni* WT 11168H, 81-176 and encapsulated *S. pneumoniae,* serotype F19. Un-treated Caco-2 cells, deficient in NOD2, were included as control. 24h post-infection, IL-8 protein levels in cell-culture supernatants were analysed by ELISA. Data is expressed as fold-induction of IL-8 protein levels compared to respective (+/-NOD2) uninfected control Caco-2 cells. Error bars indicate +/-SEM of 2 independent experiments performed in duplicates.

### 5.3.7 Intestinal epithelial NOD1 engagement mediates antimicrobial defence against *C. jejuni*

The biological consequence of NOD1-mediated antimicrobial activity on *C. jejuni* was also investigated. For this purpose, bacterial co-culture studies with WT strain 81-176 were performed over 20h on control untransfected, siNEG, siNOD1a, siNOD1b and NOD2 transfected Caco-2 cells. This time period was chosen to allow sufficient time for bacterial adhesion/invasion, host sensing and initiation of innate antimicrobial response. Following bacterial co-culture, cells were washed and subjected to a standard gentamicin protection assay. Lysed cell suspensions were diluted and viable intracellular bacteria enumerated by CFU counts on blood agar plates. Duplicates from a representative experiment are shown in Figure 5.12a (columns A & B). Data of two similar experiments is schematically shown in Figure 5.12b. At the same serial dilution status, no bacteria were detected in control cells, similarly, siNEG cells also showed minimal presence of live intracellular *C. jejuni.* Most importantly, the specific knock-down of NOD1 (siNOD1 a & b) during infection greatly enhanced the number of intracellular *C. jejuni* cells. In contrast, the additional presence of NOD2 did not appear to modulate the number of intracellular bacteria when compared to control untransfected cells.

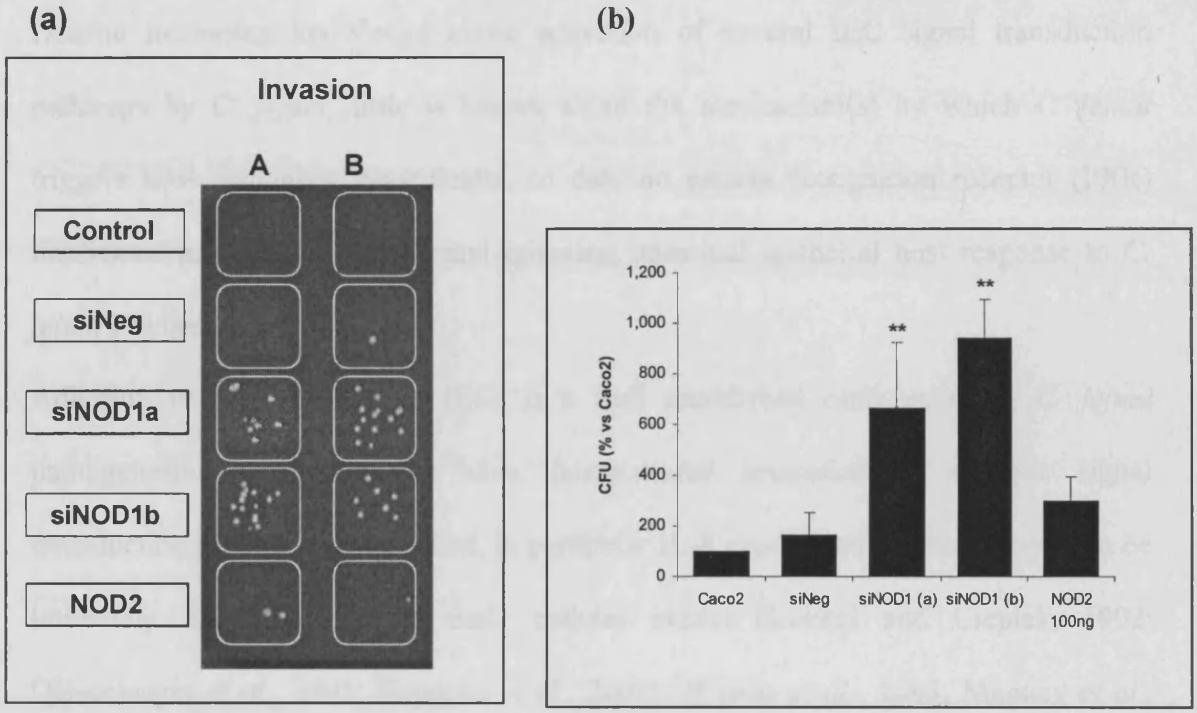



**Figure 5.12a and b: Effect of IEC NOD1 expression on the presence of intracellular *C. jejuni* cells following 20h co-culture.** Caco-2 cells were reverse transfected with siRNA (sequences a & b) targeting NOD1, siNEG or with plasmid expressing NOD2. Non-treated Caco-2 cells served as control. 48h post-transfection, cells were co-cultured with live WT *C. jejuni* 81-176 for 20h prior to enumeration of intracellular bacteria. Duplicates (columns A & B) from a representative experiment are shown (a). Statistical analysis of two to three independent experiments performed in triplicates is also shown (b). Data represents average percentage CFU obtained in treated *versus* untreated Caco-2 cells (the latter set as 100%). **, $P<0.001$ *vs.* untreated Caco-2 cells.

## 5.4 Discussion

Despite increasing knowledge about activation of several IEC signal transduction pathways by *C. jejuni*, little is known about the mechanism(s) by which *C. jejuni* triggers host responses. Specifically, to date no pattern recognition receptor (PRR) implicated in bacterial sensing and initiating intestinal epithelial host response to *C. jejuni* has been identified.

Adhesion to and invasion of IECs is a well established early event in *C. jejuni* pathogenesis. Several studies have demonstrated activation of multiple signal transduction pathways by *C. jejuni*, in particular IL-8 production has been shown to be intimately linked with these early cellular events (Konkel and Cieplak, 1992; Oelschlaeger *et al.*, 1993; Kopecko *et al.*, 2001b; Biswas *et al.*, 2003; Mooney *et al.*, 2003; Hu and Hickey, 2005; Watson and Galan, 2005). Despite the lack of complete correlation between the degree of *C. jejuni* invasion and IEC IL-8 protein induction, the process of 'invasion' itself is a crucial event involved in eliciting innate immune responses. Experiments conducted in the presence of PFA fixed bacteria showed marked absence of intracellular *C. jejuni* cells (Figure 5.3) and correspondingly, IECs exhibited weak IL-8 gene (Figure 5.1) and protein expression (Figure 5.2), thus highlighting the requirement for *C. jejuni* cell components to be within the IEC cytoplasm for optimal host detection and response.

Unlike *Salmonella* which engages host TLR5, *C. jejuni* flagellin has been found to have weak immune-stimulatory properties with minimal interaction with IEC TLR5 (Andersen-Nissen *et al.*, 2005; Watson and Galan, 2005; Johanesen and Dwinell, 2006). Armed with the information that bacterial invasion is crucial for IEC immunity, coupled with minimal involvement of TLR5 led us to hypothesize that the family of intracellular NOD receptors could be likely PRR(s) for *C. jejuni*.

Firstly we investigated if *C. jejuni* infection could modulate the expression of the potential PRR(s). Utilizing Caco-2 and HT -29 cell lines and two bacterial WT strains in co-culture experiments, we found no significant modulation of NOD1 expression in the 24h infection period (Caco-2: Figure 5.4a and b, HT-29: Figure 5.5a and b; top panel). This was an important observation as it suggests that the bacterium does not directly modulate NOD1 expression as an early immune evasion strategy. However, due to the limitations of an *in vitro* cell line model of infection, one cannot rule out bacterial-driven changes in NOD1 expression and function in an ongoing infection *in vivo.* Cytokines such as IFNγ are known to augment NOD1 expression (Hisamatsu *et al.*, 2003a) suggesting greater complexity to *C. jejuni*/epithelial NOD1 interactions in an on-going inflammatory episode are likely. In contrast, NOD2 gene expression was not detected in control Caco-2 or HT-29 cells (Figure 5.4 and 5.5; third panel) and infection with either *C. jejuni* strain did not result in NOD2 induction, suggesting that it is NOD1 and not NOD2 that maybe a potential PRR for *C. jejuni* in our model system. In the same experiments, we confirmed appropriate infectivity by following the expression of hBD-2, a peptide whose expression is NOD1 activation dependent (Boughan *et al.*, 2006). Induction of hBD-2 in the absence of NOD2 clearly implicated NOD1 as a potential intracellular sensor for *C. jejuni.*

Next, we sought evidence for a functional role for NOD1 in *C. jejuni* infection. For this purpose we transiently transfected two NOD1 siRNA sequences prior to exposure of cells to *C. jejuni.* We observed marked reduction of *C. jejuni* IL-8 and hBD-2 gene expression in cells treated with siNOD1a and b, while treatment with non-targeting siNEG did not have any effect (Figure 5.6a and b). Similarly, siNOD1 caused significant reduction of IL-8 protein levels induced by *C. jejuni* (Figure 5.7). This specific inhibitory effect of siNOD1 was confirmed at the transcriptional level using luciferase promoter assays (Figure 5.9a and b). Importantly, despite the marked knock-

down of the NOD1 gene expression (Figure 5.6a and b) its corresponding inhibitory effect on IL-8 and hBD-2 gene (Figure 5.6 a and b) and promoter function (Figure 5.7a-f) ranged between 40 and 60%, suggesting NOD1-independent signaling events are also involved in IEC innate immune gene regulation. Several studies have implicated MAP kinase pathways in *C. jejuni*-mediated IL-8 production (MacCallum *et al.*, 2005; Watson and Galan, 2005) and it is most likely that they are also operative in our model system.

Studies by Podolsky and colleagues have suggested a role for NOD2 in intestinal epithelial defence against *Salmonella* infection (Hisamatsu *et al.*, 2003b). In our study, over-expression of NOD2 by transient transfection did not augment innate defence gene expression in response to *C. jejuni* infection (Figure 5.10). To validate the functionality of transfected NOD2 in our culture system, we included live *Streptococcus pneumoniae* infection as a positive control as Optiz and co-workers have elegantly demonstrated NOD2 as the intracellular PRR for *S. pneumoniae* (Opitz *et al.*, 2004) (Figure 5.11). Why *Salmonella* and *Campylobacter*, two Gram-negative enteropathogens exhibit such contrasting requirement for NOD2 warrants further investigation.

Finally, if antibacterial immunity is generated in response to *C. jejuni* infection *via* NOD1 activation (e.g. hBD-2 production), one can hypothesise that absence of this armory is likely to enhance bacterial invasion and/or survival. We followed the biological consequence of NOD1 knock down on *C. jejuni* infection. We found marked increase in the number of invasive bacteria 20h post-infection in cells treated with siNOD1a or b sequences (Figure 5.12a & b), suggesting unequivocally that NOD1 mediated cellular events do contribute to the number of intracellular bacteria, whether this is due to increased invasion or increased survival or both remains a question for future work.

The identification of NOD1 as a major sensor to enteroinvasive *E. coli* led Kagnoff and colleagues to propose that NOD1 signaling in the IEC may provide a backup mechanism for rapidly activating innate immunity during infection by highly invasive pathogenic Gram-negative enteropathogens (Kim *et al.*, 2004). Our study implicating NOD1 as a major intracellular PRR for *C. jejuni*, lends further support for a critical role for NOD1 mediated immunity at the gastrointestinal mucosal surface. Further studies are now required to investigate *C. jejuni* mediated signaling *via* NOD1 in more detail. Activation of both TLRs and NOD-like receptors (NLRs) by different bacterial PAMPs is emerging as an important factor in mounting the hosts inflammatory response and priming of cells with PGN resulting in activation of NLRs has a synergistic effect on TLR signaling (Kufer and Sansonetti, 2007). Insight into mechanisms involved in *C. jejuni* PGN delivery, the subsequent recognition by NOD1 and possible interplay between NLR and TLR signaling pathways will shed greater light on the role of *C. jejuni* invasion in eliciting host responses.

# CHAPTER 6

# *Ex-vivo* model to study *C. jejuni* disease pathogenesis

## 6.1 Introduction

Although intestinal pathology caused by *C. jejuni* in humans can vary, ranging from mild non-inflammatory watery, to severe bloody diarrhoea (Blaser, 1997), the presence of fresh blood, pus or mucous suggesting the presence of colorectal inflammation remains a common feature of infection (Wassenaar and Blaser, 1999; Crushell *et al.*, 2004). Despite the clinical spectrum described, our knowledge of disease pathogenesis at molecular level remains limited. One of the main reasons for this is the lack of a suitable animal model, as *C. jejuni* colonizes most domestic and wild animals in an apparently commensal association with little or no pathology (Shreeve *et al.*, 2000). Furthermore, due to the generally mild and self-limiting nature of disease, hospitalization is rarely required and endoscopic examination during acute infection is rarely performed. Limited reports of colonoscopy (i.e. sigmoidoscopy) findings have revealed mucosal changes in the large bowel ranging form oedema and hyperaemia, petechial haemorrhages and mucosal friability (van Spreeuwel *et al.*, 1985; Ketley, 1997). These changes can sometimes be difficult to distinguish from those observed in patients suffering from IBD, such as Ulcerative Colitis (van Spreeuwel *et al.*, 1985; Siegal *et al.*, 2005). Inflammation can also occur in parts of the ileum and jejunum commonly associated with mesenteric adenitis (Loss *et al.*, 1980; Ketley, 1997; Wassenaar and Blaser, 1999). Collectively, data suggest that *C. jejuni* can cause inflammation in both the small and the large bowel. However, at present the sequence of adhesion and colonization, possible cell type tropism (e.g. Peyers Patches, Goblet cells) and early host pathogen interactions *in-vivo* remain largely unknown. Despite several advantages of the use of IEC lines as a reliable and reproducible *in-vitro* model to study *C. jejuni*–epithelial interactions, limitations of the model are clear. These include the absence of other cell types (i.e. Paneth cells, goblet cells), which interact in a complex three-dimensional manner leading to a concerted intestinal response to *C.*

*jejuni*, absence of a thick mucus layer and differences in the structural function of the various parts of the digestive tract.

To gain a better understanding of early *C. jejuni* – intestinal interaction, in this series of experiments we initially wished to establish an *ex-vivo* model of infection. Human intestinal forceps biopsy samples from small and large bowel of children undergoing lower endoscopy were obtained and cultured for up to 12h in the presence of *C. jejuni*. Following infection, the tissue was fixed and host pathogen interaction studied by immunostaining and confocal microscopy.

A second major limitation of *in-vitro* and *ex-vivo* models used to study GI bacterial host pathogen interactions, is that culture conditions generally favour eukaryotic survival. Specifically, high oxygen levels are required to ensure host cell survival while many enteric bacteria (commensals and pathogens) including *C. jejuni* are adapted to microaerophilic/ anaerobic conditions. In the GI tract, oxygen supply for the intestinal epithelium is provided *via* the underlying submucosal capillary network, while conditions in the lumen and the apical epithelial surface are anaerobic. One may hypothesize, that bacteria/pathogens in their natural anaerobic environment are likely to express virulence genes whose expression may differ in aerobic, general laboratory conditions. Taking these factors into account, we aimed to design a model of infection closely mimicking conditions in the GI tract. A vertical diffusion chamber system was employed to provide ideal growth conditions for both *C. jejuni* and host IECs. Following optimization, experiments were performed to test for potential differences in virulence according to culture conditions.

## 6.2 Methods

### 6.2.1 *In-vitro* organ culture (IVOC) of intestinal biopsies

All reagents were supplied from Sigma, Poole, UK unless stated otherwise.

6.2.1.1 Preparation of IVOC medium

IVOC medium was prepared by adding 0.95g powdered NCTC-135 media and 1g D-mannose (to prevent non-specific adherence) to 100ml sterile, deionised water. Following, pH was adjusted to 7.4 by adding 0.22g sodium bicarbonate. Finally, 100mls of tissue culture medium (DMEM+ Glutamax) and 20mls FCS were added to 100mls of filter sterilized NCTC-135 media (0.22μm filter, Millipore) to make up complete IVOC medium.

6.2.1.2 IVOC protocol and bacterial co-culture

This method has previously been established for the co-culture of intestinal biopsies with *E. coli* (Hicks *et al.*, 1996). Histologically normal mucosal samples from ileum and colon were obtained from pediatric patients undergoing endoscopic investigation. Institutional ethical approval (ID: BRD/06/049) and parental consent were obtained. Immediately after collection, samples were transferred into IVOC medium and transported to the laboratory. Biopsies were examined for tissue integrity under a dissection microscope (XTL-101, GX Optical, Suffolk, UK), and mounted (mucosa side up) on sponge support units (autoclaved sponge rubber pieces) placed in 12 well plates and soaked in complete IVOC medium. Fluid levels in each well were adjusted to provide partial submersion of biopsies in IVOC medium (Figure 6.1a).

Twenty to fifty microliters of bacterial suspension containing approximately $10^8$ CFU (*C. jejuni* 11168H) was applied immediately to the apical/mucosal surface of tissue samples. An uninoculated specimen was included in each experiment as a negative control. Co-cultures were placed on a rocking platform/shaker and maintained at 37°C and 5% $CO_2$ in a humidified incubator (Galaxy $CO_2$ Incubator, Wolf Laboratories, UK)

for up to 12h (Figure 6.1b). Tissue culture medium was changed regularly (2-3h) to maintain pH and nutrient levels and additional *C. jejuni* suspension applied twice. Following incubation period, tissue samples were washed 3 times in fresh tissue culture medium to remove any non-adherent bacteria, transferred into 4% PFA stored at 4°C until further processing.

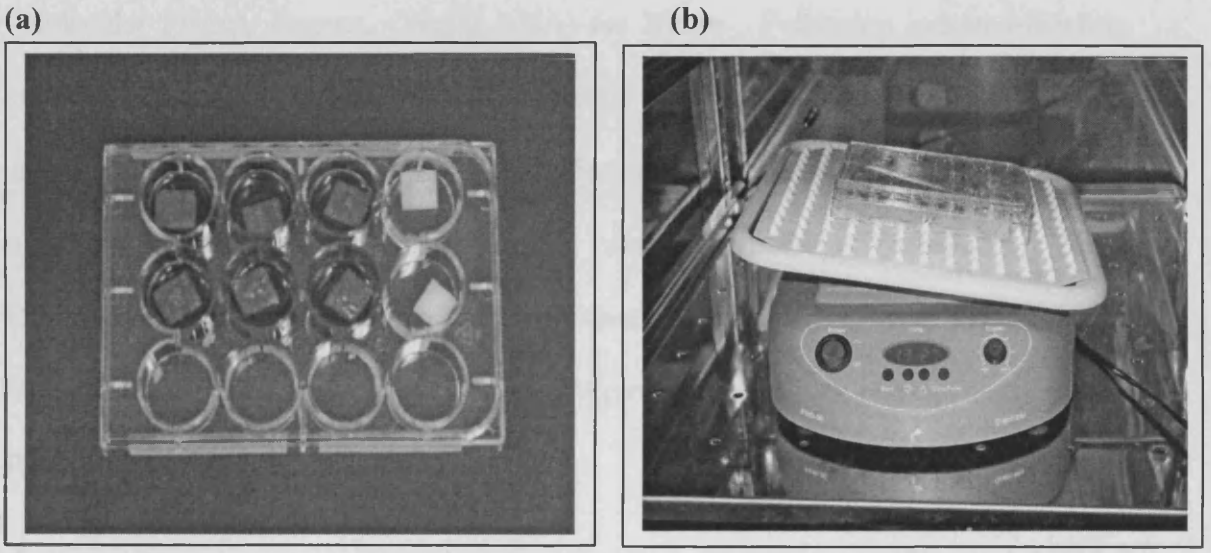



**Figure 6.1a and b: *In-vitro* organ culture of intestinal forceps biopsies.** Intestinal biopsies from terminal ileum and colon were mounted on sponge support units placed in 12 well plates and soaked in IVOC medium mucosal side facing up (a). Twenty to fifty microliters of *C. jejuni* in suspension was directly applied to biopsies and samples maintained on a rocking platform at 37°C and 5% $CO_2$ in a humidified incubator (b).

### 6.2.2 *C. jejuni* immuno-labeling and biopsy staining

4% PFA-fixed tissue was washed three times in PBS to remove residual traces of PFA. Tissue was lysed by immersion in 0.2% Triton X 100/PBS solution for 5min at RT. Non-specific binding was blocked by incubating tissue samples in 1% BSA/PBS for 45min. *C. jejuni* was labeled using primary unconjugated goat anti-*C. jejuni* antibodies (KPL, Maryland, USA) at a concentration of 5μg/ml for 60min at RT, followed by incubation with FITC-conjugated rabbit anti-goat IgG (0.5μg/ml, Molecular Probes, Eugene, Oregon, USA) for 30min. Cell nuclei were counterstained with TO-PRO-3 (Molecular Probes, Eugene, Oregon, USA) for 30min. Following immuno-labeling, actin cytoskeleton was stained with 1Unit/ml rhodamine phalloidin in PBS/BSA solution (Invitrogen Ltd, Paisley, UK) for 45min. After each step, cells were washed 3 times with PBS. Finally, whole biopsies were mounted in Vectashield (Vector Laboratories Ltd., Peterborough, UK) and visualized with a Radiance 2100 confocal laser scanning microscope equipped with an Argon-Krypton laser and a red diode (Bio-Rad Laboratories, Hemel Hempstead, UK).

### 6.2.3 Tight junction staining of Caco-2 cell monolayers

Occludin is an integral transmembrane protein, associated with epithelial tight junctions (Kimura *et al.*, 1997). In order to monitor tight junction formation and monolayer integrity, Caco-2 cells grown on snapwell inserts (diameter: 12mm, pore size: 0.4 μm; Fisher Scientific, Leicestershire, UK) were stained for occludin. Briefly, approximately 4 x $10^5$ cells were placed into each snapwell insert and grown over a minimum of 21d to allow for polarization and tight junction formation. Inserts were placed into vertical diffusion chamber devices and incubated for 20h in either $CO_2$ or VAIN incubator (will be described in 6.3.2). Post-incubation, cells were fixed in 4% PFA and lysed for 5min at RT in 0.2% Triton X 100/PBS. Following blocking of non-

specific binding sites in BSA, cells were incubated in 10μg/ml unlabeled polyclonal rabbit anti-occludin antibody (ZYMED Laboratories, San Francisco, USA) for 1h at RT. FITC labelling was performed by incubation in secondary anti-rabbit antibody (0.5μg/ml) for 30min at RT (Molecular probes, Eugene, Oregon, USA). Cell nuclei were counterstained with TO-PRO-3 (Molecular probes, Eugene, Oregon, USA).

## 6.3 Results

### 6.3.1 *C. jejuni* interactions with human intestine

Prior to investigating *C. jejuni* cross-talk, tissue survival in the organ culture system over a period of up to 12h was studied. As shown in Figure 6.2, uninfected samples from both small (terminal ileum) and large bowel (colon) remained structurally intact with the outline of small intestinal villi (Figure 6.2a) and colonic crypts (Figure 6.2b) visible. Following demonstration of tissue survival/integrity, co-culture experiments of intestinal biopsies with WT *C. jejuni* 11168H were performed and host-pathogen interactions analyzed by immuno-labelling of *C. jejuni* and confocal microscopy.

Although the majority of *C. jejuni* organisms were found in the mucus layer covering the epithelial surface in the terminal ileum (Figure 6.3a and b), close contact between *C. jejuni* and the intestinal epithelium was also observed at several sites in the same biopsy (Figure 6.3c and d). This was a reproducible and regular finding in biopsies infected with *C. jejuni* 11168H for more than 8h. Interestingly, despite the close contact between *C. jejuni* and the epithelium, the brush border appeared to remain intact with no visible actin rearrangements (Figure 6.3c and d). An intriguing finding was the presence of small clusters of *C. jejuni* that appeared to associate with single cells of the terminal ileum (6.4a and b). These findings have been noted in only one sample. In contrast to our observations in small bowel, to date similar *C. jejuni* localisation in the colon has not been found.

(a) (b)

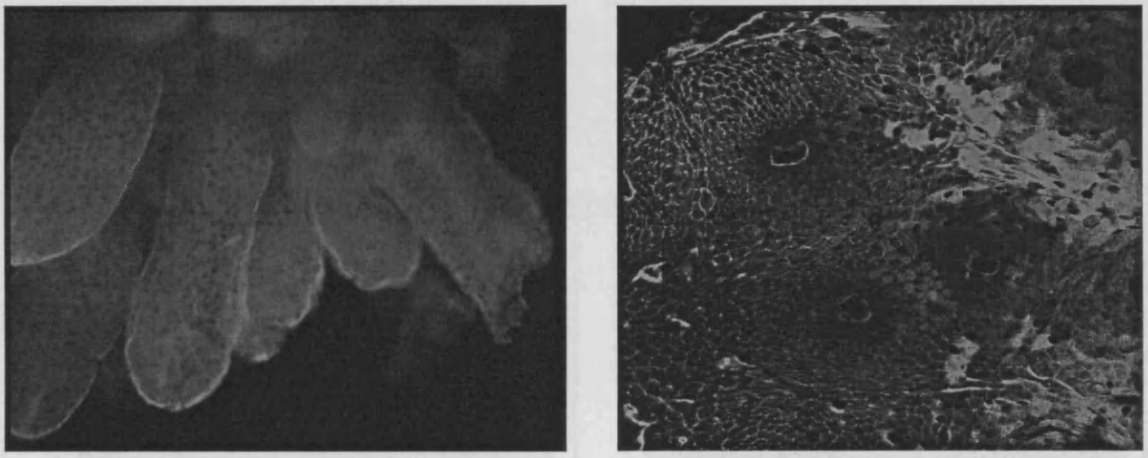


**Figure 6.2a and b: Integrity of intestinal biopsies following 12h *ex-vivo* culture.** Intestinal forceps biopsies of terminal ileum (a) and colon (b) were incubated in IVOC media over 12h. Following fixation in 4% PFA, actin cytoskeleton was stained with rhodamine phalloidin (red) and nuclei counterstained with TO-PRO3 (blue). Intact small intestinal villi (a) and colonic crypts (b) as observed by confocal microscopy.

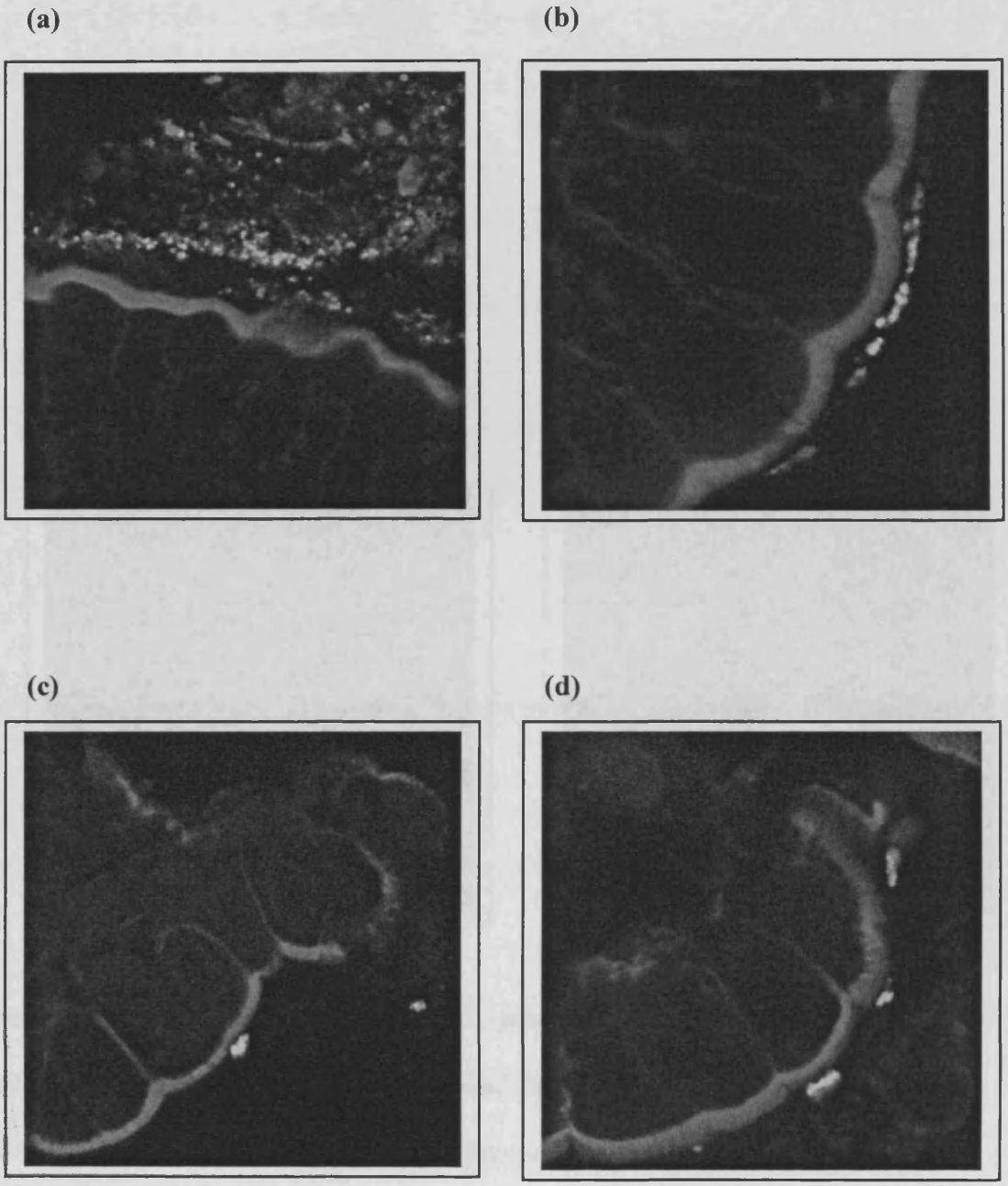



**Figure 6.3a to d: Co-culture of *C. jejuni* with human small bowel biopsies.** Human intestinal biopsies from the terminal ileum were co-cultured for 12h with WT *C. jejuni* 11168H. Post-infection, bacteria (green) and actin filaments including apical brush border (red) were visualized. TO-PRO3 (blue) allowed nuclear staining. Whole tissue samples were mounted on glass slides and examined by confocal microscopy.

(a) (b)

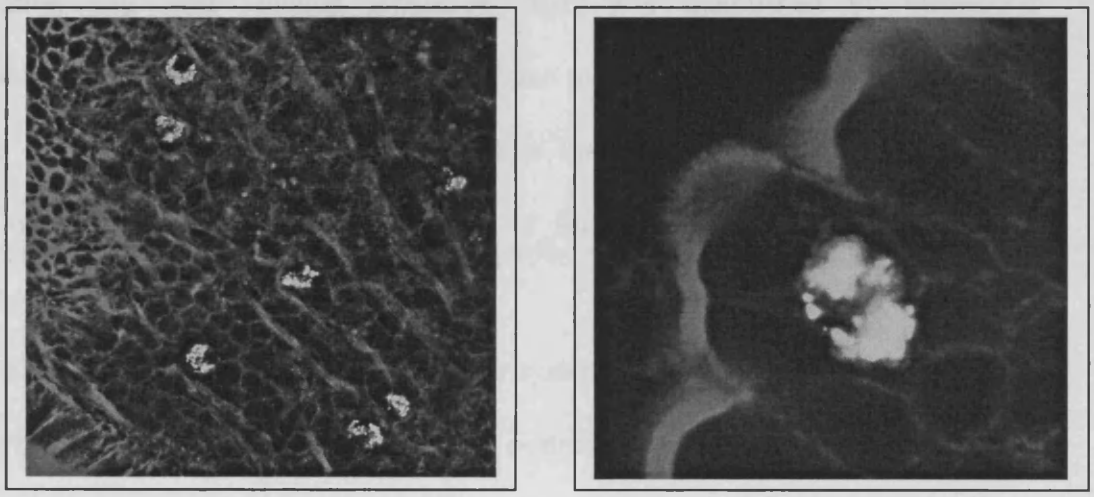


**Figure 6.4a and b: Small clusters of *C. jejuni* associated with small bowel epithelium.** Intestinal biopsies from the terminal ileum were co-cultured with *C. jejuni* 11168H for 12h and bacteria visualized by immunolabelling (green). Actin filaments were stained with rhodamine phalloidin (red). Biopsies were mounted on coverslips and examined by confocal microscopy.

### 6.3.2 The asymmetrical vertical diffusion chamber system

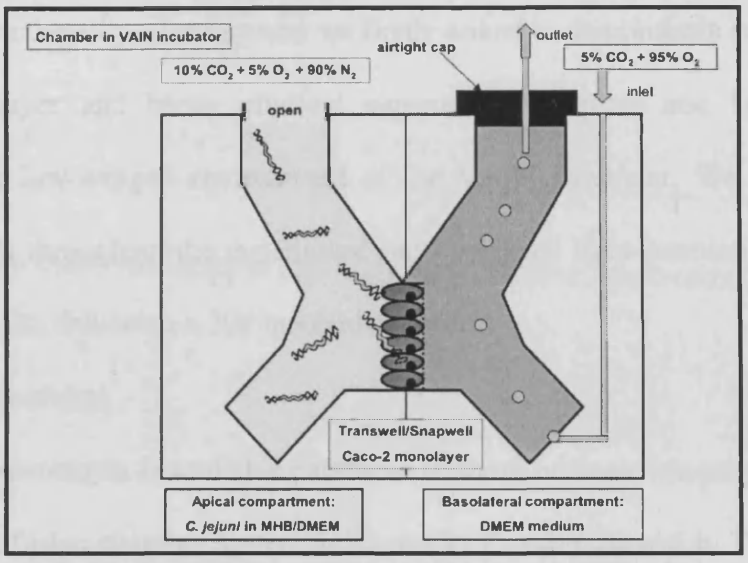
The diffusion cell-system, derived from an Ussing chamber, was originally developed by Grass and Sweetana to study the transport of substances across membranes (Grass and Sweetana, 1988). In the present study, a vertical diffusion chamber system (Harvard Apparatus Ltd, Kent, UK) was utilised to provide optimal growth conditions for both *C. jejuni* and Caco-2 cells (Figure 6.5).

Caco-2 cells were grown on snapwell filters over a minimum of 21 days to allow for polarization and tight junction formation. This was established by measuring transepithelial electrical resistance (TEER) prior to experiments using a DVC-1000 voltage clamp (details below; World Precision Instruments, Hertfordshire, UK). A TEER of >200 ohm x $cm^2$ was indicative of formation of a tight, polarized cell monolayer.

6.3.2.1 Set up of the vertical diffusion chamber system

The vertical diffusion chamber was set up as outlined in Figure 6.5. Polarized Caco-2 cell monolayers on snapwell inserts were placed into the diffusion chamber device creating an apical and basolateral compartment, which were filled with tissue culture medium. Several chambers could be mounted serially allowing for inclusion of control and duplicate samples. The chambers were placed into a variable atmosphere incubator (VAIN, Don Whitley Scientific Ltd, Shipley, UK) containing 90% $N_2$, 5% $O_2$ and 10% $CO_2$ at 37°C and hence providing ideal growth conditions for *C. jejuni*. The apical compartment was left open and exposed to the microaerobic atmosphere within the VAIN, whilst the basolateral compartment was closed with airtight caps. Basolateral oxygen supply for Caco-2 cells was provided by constant administration of a gas mixture containing 5% $CO_2$ and 95% $O_2$. The gas mixture flow rate was set to approximately 5-10mls/min (30-40 gas bubbles/min) with an outlet tube preventing accumulation of pressure.

(a)




(b)

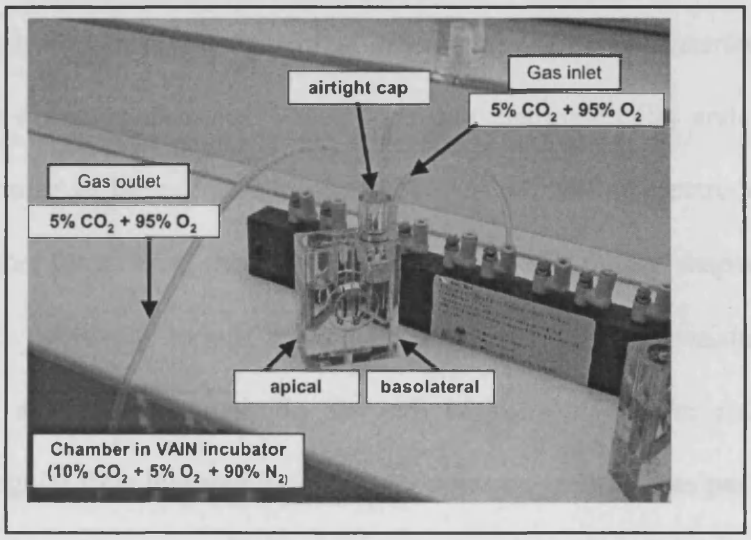



**Figure 6.5: Asymmetrical, vertical diffusion chamber system.** A vertical diffusion chamber system was set up as illustrated schematically (a) and in the laboratory (b), to provide optimal culture conditions for both *C. jejuni* and Caco-2 cells. Differentiated Caco-2 cell monolayers grown on snapwell inserts were placed in the diffusion cell, creating an apical and basolateral compartment. With the apical compartment left open, the chamber was placed in a VAIN incubator containing 10% $CO_2$, 5% $O_2$ and 90% $N_2$. The basolateral compartment was closed with an airtight cap and continuously gassed with a mixture containing 5% $CO_2$ and 95% $O_2$. A gas outlet tube, leading out of the VAIN incubator, prevented accumulation of gas pressure.

6.3.2.2 Assessment of monolayer integrity in VAIN incubator

In order to prove functionality of our system we firstly aimed to demonstrate survival of Caco-2 cell monolayer and hence physical separation of apical and basolateral compartment in the low oxygen environment of the VAIN incubator. We therefore monitored the TEER throughout the experiment and visualized tight junction integrity by staining for occludin following a 20h incubation period.

a) TEER measurements

TEER across a cell monolayer is a reliable parameter to monitor tissue integrity and was performed in our diffusion chamber system as shown in Figure 6.6a and b. Two silver chloride (AgCl) electrodes (Harvard Apparatus Ltd, Kent, UK) were inserted in each compartment of the diffusion chamber. Voltage sensing electrodes ($V_1$ and $V_2$) were placed in close proximity of the cell monolayer, while current passing electrodes ($I_1$ and $I_2$) were placed further away form the tissue (Figure 6.6a). An empty snapwell insert (without Caco-2 cells) was used to auto zero and compensate for fluid resistance prior to the insertion of a snapwell containing the cell monolayer. At the start of the experiment and at regular time intervals an electrical current (180μA) was passed via $I_1$ and $I_2$ and voltage read-out on the voltage clamp recorded. Resistance was calculated according to Ohm's law: R (in Ω)=V / I and monolayer TEER (in $\Omega cm^2$) calculated by multiplying resistance with the surface area of the snapwell insert (1.12 $cm^2$). As shown in Figure 6.6c, TEER in cells maintained in the VAIN incubator over a 20h time period remained above 200 $\Omega cm^2$, suggesting integrity of cell monolayer.

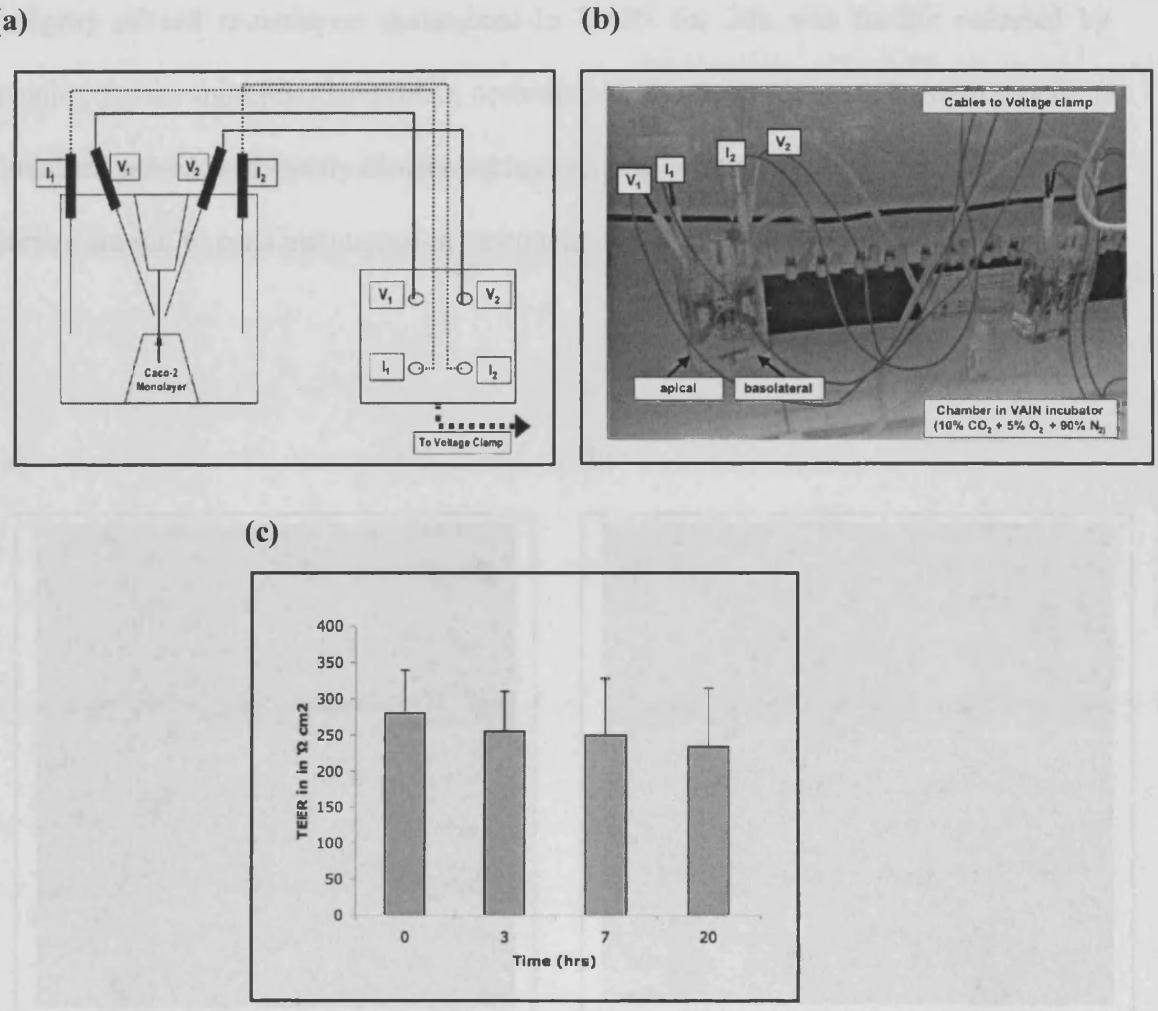



**Figure 6.6a to c: Measurement of TEER in the vertical diffusion chamber system.** Two AgCl electrodes were placed in each compartment, with voltage sensing electrodes placed in close proximity to the cell monolayer ($V_1$ and $V_2$). Electrodes were connected to the voltage clamp as outlined in (a). A current of 180μA was passed via $I_1$ and $I_2$ and voltage read-out recorded. Resistance was calculated according to Ohm's law and TEER (in Ω $cm^2$) obtained by multiplication with the surface area of snapwell inserts (1.12 $cm^2$). (c) TEER measurements of Caco-2 monolayer maintained in VAIN for 20h. Arrow bars indicate mean +/- SEM of one experiment performed in duplicates.

b) Assessment of monolayer integrity by tight junction staining

Integrity of cell monolayers maintained in VAIN for 20h was further assessed by staining for the tight junction protein occludin. As shown in Figure 6.7a, tight junctions remained present and evenly distributed between the cell borders, with occludin staining pattern similar to cells maintained in air containing $CO_2$ tissue culture incubator (b).

(a) (b)

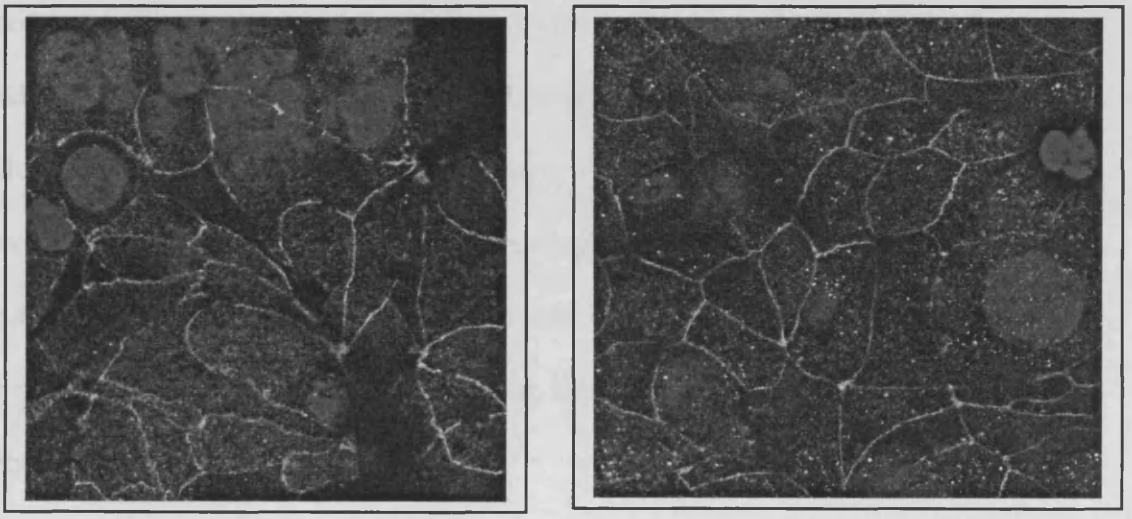


**Figure 6.7a and b: Assessment of tissue integrity by staining for tight junctions.** Caco-2 cell monolayers maintained in the vertical diffusion chamber system for 20h. Tissue integrity was assessed by staining for the tight junction protein occludin (green) and distribution of tight junctions in VAIN (a) compared to cells maintained in a $CO_2$ incubator (b). Cell nuclei were counterstained with TO-PRO-3 (blue).

6.3.2.3 Bacterial growth, adhesion and invasion in VAIN, preliminary results

Following demonstration of Caco-2 survival and monolayer integrity, *C. jejuni* virulence in low oxygen condition was investigated by assessing bacterial growth, adhesion and invasion using a gentamicin protection assay. Approximately 3 x $10^6$ CFU of *C. jejuni* WT 11168H were added to the apical compartment of the diffusion chamber and placed either in the VAIN or $CO_2$ incubator. Following 3.5h incubation period, bacterial growth and adhesion were assessed by plating out serial dilutions of supernatants and cells lysates respectively on blood agar plates. In a second chamber, media in both compartments was replaced with media containing 150μg/ml gentamicin and cells incubated for 60min. Bacterial invasion was assessed by plating out serial dilutions of cell lysates on blood agar plates followed by counting of CFU three days later. As shown in Figure 6.8, we observed higher rates for bacterial growth, adhesion and invasion for *C. jejuni* 11168H kept in low oxygen VAIN for 3.5h when compared to normal oxygen $CO_2$ incubator. However, this is data from one experiment only and requires confirmation.

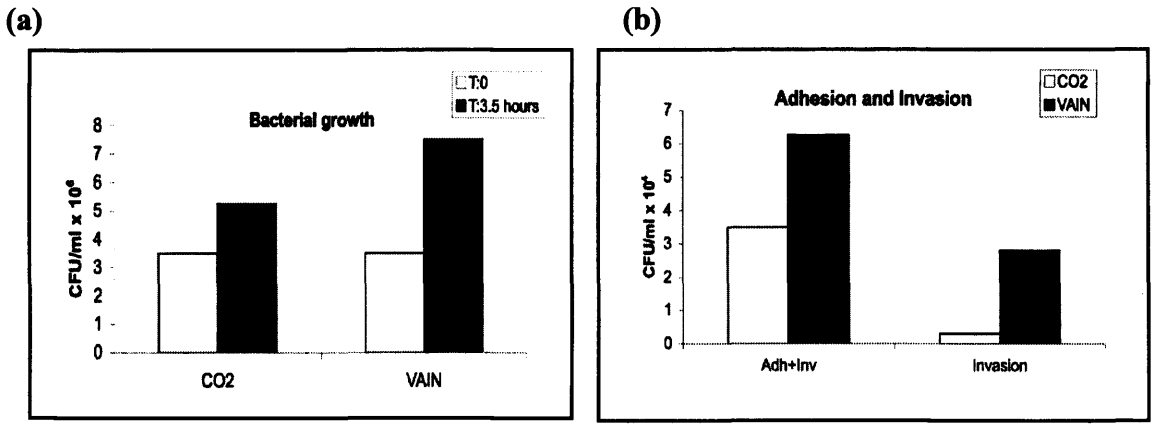



**Figure 6.8a and b: *C. jejuni* 11168H growth, adhesion and invasion in VAIN *versus* $CO_2$ incubator.** Approximately 3 x $10^6$ CFU of WT *C. jejuni* 11168H were placed in the apical compartment of the vertical diffusion chamber containing Caco-2 cell monolayers. Two diffusion chambers each were placed in VAIN and $CO_2$ incubator for 3.5h. Following, bacterial growth/survival (a), adhesion and invasion (b) were assessed using a standard gentamicin protection assay.

## 6.4 Discussion

Our knowledge about early host pathogen interactions between *C. jejuni* and the human intestinal mucosa *in-vivo* remain limited. Reasons include the lack of a suitable animal model and the generally mild, self-limiting nature of human intestinal disease. Additionally, several major limitations of cell-line models currently available underline the need for novel infection models to study *C. jejuni* disease pathogenesis.

For data presented in this chapter, we used paediatric intestinal forceps biopsies obtained from healthy children as an *ex-vivo* model of infection to investigate early host-*C. jejuni* interactions. Since the distribution and pattern of intestinal colonization by *C. jejuni* is unknown, we aimed to shed further light on which part of the digestive tract is initially targeted by the bacterium. Co-culture studies on both the small (terminal ileum) and large bowel (colon) with WT *C. jejuni* 11168H were performed.

Unlike in cell line models (i.e. Caco-2 cells), where bacteria have direct access to the epithelium allowing for adhesion and invasion to occur, the intestinal tissue is covered in a thick mucus layer. As an important initial observation, we found the majority of *C. jejuni* to be localized within the mucus film overlaying the intestinal epithelium of small bowel tissue (Figure 6.3a and b). These findings are consistent with studies by Grant and colleagues, suggesting that *C. jejuni* preferentially associates with mucus overlying tissue of duodenal biopsies (Grant *et al.*, 2006). Furthermore, several studies have provided evidence that *C. jejun* is chemotactic towards mucin and may explain the localisation of majority of *C. jejuni* found in our study (Figure 6.3a and b). Taking into account that ingestion of only a few hundred *C. jejuni* organisms can cause disease in humans (Black *et al.*, 1988), one may hypothesise that the bacterium colonizes the mucus layer, allowing for multiplication and expression of virulence genes prior to further progression and interaction with the intestinal epithelium.

As a consistent finding, we observed *C. jejuni* in close association with the small bowel epithelium (Figure 6.3c and d). Importantly, these areas were commonly overlaid by *C. jejuni* containing mucus, lending further support for the hypothesis that the bacterium colonizes the mucus layer prior to adhesion and invasion of the IEC. However, despite the close contact of *C. jejuni* with the epithelial layer, the brush boarder remained intact with no visible actin rearrangements. Furthermore, in contrast to well-established findings in cell line models, we were unable to demonstrate bacterial invasion in our *ex-vivo* system. However, this might be due to the relatively short incubation period of 12h, which might not be sufficient for bacterial invasion to occur.

We observed several clusters of *C. jejuni* associated with single cells within the small bowel epithelial layer in one biopsy sample (Figure 6.4a and b). Unfortunately we were unable to verify the cell type and potential cell topism remains speculative. However, similar findings were observed in the duodenum by Grant and colleagues suggesting that these cells might be mucus producing goblet cells (Grant *et al.*, 2006). Experiments are currently in progress to address these questions.

In contrast to our findings in small bowel biopsies, we were unable to demonstrate similar close association of *C. jejuni* with the intestinal epithelium in the colon. Whether this is due to limitations of our IVOC model and imaging techniques or a true reflection of *in-vivo* events remains unanswered. However, if this was a true event, this finding stands in some contrast to observations from human infection, stating that the major inflammatory changes occur in the colon (Blaser, 1997). One possible explanation would be that *C. jejuni* might initially target the small bowel before colonizing the large intestine. This process can be expected to take longer than the studied 12h period and therefore might help to explain the absence of *C. jejuni* in colonic tissue samples in our system. However, limitations of the IVOC system have to be considered when interpreting these results. Most importantly, culture conditions in

the $CO_2$ tissue culture incubator (high oxygen), were suboptimal for *C. jejuni* and in favour of the eukaryotic cell. With *C. jejuni* requiring microaerophilic conditions for ideal growth, high oxygen might prevent the bacterium from exhibiting its full virulence and hence mask important events occurring *in-vivo*. To address this problem, we utilised a vertical diffusion chamber system to provide ideal growth conditions for both *C. jejuni* and intestinal epithelial Caco-2 cells (Figure 6.5).

Optimization of system set-up included demonstrating survival of Caco-2 cells in low oxygen within the VAIN. This was important as integrity of the cell monolayer is mandatory to ensure the physical separation of apical and basolateral compartments and hence separation of both gas environments. We therefore monitored monolayer integrity over a 20h incubation period by measuring TEER throughout the experiment (Figure 6.6) and visualized tight junction integrity by staining for occludin (6.7). With TEER remaining above 200 $\Omega$ $cm^2$ (Figure 6.6c) and staining for the tight junction protein occludin suggesting their integrity (Figure 6.7a), we were able to provide good evidence not only for tissue survival but also successful separation of apical and basolateral compartments. Unfortunately, due to technical limitations and lack of facilities (i.e. blood gas analyzer) we were unable to measure exact oxygen concentrations in apical and basolateral compartments, however, the constant measurement and adjustment of $CO_2$ and $O_2$ levels to set values by the VAIN incubator, provides further evidence for the functionality of our system.

In 2002, Cottet and colleagues published their findings on the use of a similar vertical diffusion chamber system to study *H. pylori* virulence in low oxygen conditions (Cottet *et al.*, 2002). They were able to demonstrate not only increased survival of *H. pylori* in 5% $O_2$ as compared to normal air, but also showed a significant increase in adherence to and invasion of Caco-2 cells. Following optimization of our system, we aimed to investigate differences in virulence of *C. jejuni* according to the gas environment. We

therefore performed gentamicin protection assays and analyzed bacterial survival, adhesion and invasion. Preliminary results obtained were promising, demonstrating slightly increased survival, adhesion and invasion in low oxygen conditions after 3.5h (Figure 6.8). However, these experiments were performed only once and require further confirmation. With set-up now completed, longer time periods can be investigated and parameters such as MOI, bacterial strain and infection media used in the apical compartment (i.e. tissue culture medium vs. MH broth) explored. Furthermore, we aim to replace Caco-2 cells with intestinal biopsies allowing to combine both infection models and create a novel system closely reflecting human *in-vivo* conditions.

# CHAPTER 7

# Discussion

Since its discovery a century ago, the Gram-negative bacterium *C. jejuni* has emerged as the worlds leading cause of bacterial mediated diarrhoea. In several developed countries the number of reported *C. jejuni* enteritis cases currently exceeds 80 per 100,000 people (Friedman *et al.*, 2000), while the true incidence including un-reported cases are likely to be much higher with estimates suggested in the region of 1.000 to 2.000 cases per 100,000 people (Samuel *et al.*, 2004). Numbers are even higher in developing countries where infection is endemic, reaching estimated 40,000 to 60,000 cases per 100,000 (Oberhelman and Taylor, 2000). Mainly young children under the age of 5 years are affected and with dehydration occurring rapidly in this age group, even mild watery diarrhoea can turn into a live threatening event if adequate treatment is delayed. Further, it is now well established that *C. jejuni* infection is associated with a wide range of intestinal and extra-intestinal complications. A prime example is the frequently occurring GBS (1 in 1000 cases of *C. jejuni* enteritis), an acute post-infectious ascending paralysis that can affect peripheral and cranial nerves, in severe cases requiring artificial ventilation (Douglas and Winer, 2006; Kuwabara, 2007). Despite this significant health burden caused by *C. jejuni* our knowledge about disease pathogenesis remains ill defined. However, elucidation of the genome sequence of *C. jejuni* strain NCTC11168 in recent years has accelerated research in *Campylobacter* genetics, microbiology and pathogenesis (Parkhill *et al.*, 2000a). Significant findings include the discovery of both an *O*- and *N*-linked glycosylation system allowing post translational modification of several surface structure (Szymanski and Wren, 2005), one of which is a previously un-noted capsular polysaccharide (Karlyshev and Wren, 2001). These and other features of *C. jejuni* are likely to not only modulate bacterial virulence and survival, but also influence host-pathogen interactions and disease outcome. With the possibility of creating knock out mutants now available, exciting opportunities have opened to unravel molecular events involved in *C. jejuni*-associated disease.

The variable clinical picture of *C. jejuni* enteritis and lack of direct correlation with strain virulence underlines the crucial role of host immune response(s) in determining disease severity and outcome. Furthermore, given the generally self-limiting nature of infection, insight into immune defence mechanisms that allow successful bacterial clearance are essential to further our understanding of *C. jejuni* disease pathogenesis. As the first site of host-pathogen interaction, the epithelial lining of the GI tract not only provides a crucial physical barrier, protecting the underlying mucosa from the external environment but also actively participates in microbial sensing and mounting appropriate innate immune responses by the production of chemokines, cytokines and AMPs (Kagnoff and Eckmann, 1997; Eckmann, 2005). Members of the latter group are β-defensins, evolutionary conserved small cationic peptides (Pazgier *et al.*, 2006). The now well established unique role in host defence results from their antimicrobial properties against a wide range of microbes (including bacteria, fungi, yeast and viruses) combined with their ability to act as multieffector molecules capable of enhancing innate immune response as well as activating cells of the adaptive immune system (Selsted and Ouellette, 2005; de Leeuw and Lu, 2007).

Several studies have demonstrated how defensins and their modulation *in-vivo* can influence both susceptibility to infection (Goldman *et al.*, 1997; Schroder and Harder, 1999) or conversely provide protection for the host (Salzman *et al.*, 2003). The first aim of this study was therefore to investigate the role and regulation of hBDs during *C. jejuni* infection. To date the most extensively studied β-defensins are hBD-1, -2 and -3. While hBD-1 is expressed constitutively in IEC of the GI tract, hBD-2 and -3 are inducible during infection and inflammation (O'Neil *et al.*, 1999; Ogushi *et al.*, 2001; Wehkamp *et al.*, 2003b). We firstly examined the modulation of hBD-1, -2 and -3 during *C. jejuni* infection in two frequently used intestinal epithelial cell lines Caco-2 and HT-29. We found expression of hBD-1 to remain constitutive during infection with

two virulent WT *C. jejuni* strains tested 11168H and 81-176 (Figure 3.1). This was an important finding, particularly in the light of other studies that have demonstrated up- and down-regulation of hBD-1 expression during infection with *H. pylori* and *Cryptosporidium parvum* respectively (Bajaj-Elliott *et al.*, 2002; Zaalouk *et al.*, 2004). Unlike these enteropathogens, *C. jejuni* does not seem to regulate expression of hBD-1 highlighting the distinct differences amongst microbes in modulating innate host defence. In contrast to hBD-1, we were the first to demonstrate induction of hBD-2 and hBD-3 by WT *C. jejuni* (Figure 3.1) suggesting a potential role of these peptides in disease pathogenesis. Additionally, several studies have demonstrated induction of the potent neutrophil attractant IL-8 in response to *C. jejuni* (Hickey *et al.*, 1999; Hickey *et al.*, 2000; MacCallum *et al.*, 2005; Watson and Galan, 2005). This cytokine is thought to be involved in disease pathogenesis by initiating a local inflammatory process in the affected intestinal mucosa, contributing to bacterial clearance *in-vivo (Watson and Galan, 2005)*. We were able to confirm and further extend these findings by demonstrating induction of IL-8 by *C. jejuni* 11168H and 81-176 in both cell lines tested (Figure 3.1). Interestingly, kinetics and magnitude of IL-8 gene expression followed a similar pattern as hBD-2 and hBD-3 with up-regulation occurring at 6h post infection and levels remaining elevated throughout the tested 24h time interval (Figure 3.1). These findings suggest common signaling pathway(s) involved in the regulation of several innate defence genes.

Despite lack of significance, we found a reproducible difference in innate defence gene modulation, depending on WT strain and cell line utilized. Specifically, we consistently observed slightly higher levels of β-defensin and IL-8 induction by WT strain 81-176. These differences are not surprising, considering the large genetic and microbiological diversity of *C. jejuni* strains, which is likely to influence their virulence and hence host response. In fact several studies have demonstrated differences in the ability of various

*C. jejuni* strains to induce IL-8 secretion (Hu and Hickey, 2005; Watson and Galan, 2005). Regarding the two WT strains used in this study, 81-176 is considered to be more invasive and harbors a plasmid (pVir) that has been shown to be associated with virulence (Bacon *et al.*, 2000). Experiments to compare the invasive potential of strain 11168H and 81-176 are underway (Mr. A. Elmi, LSHTM), and might provide further explanation for our findings. Additionally, differences in host response are also expected to vary according to host cells used (MacCallum *et al.*, 2006). We have observed a consistently lower induction of hBD-3 mRNA levels in HT-29 cells when compared to Caco-2 (Figure 3.1). This was again the case for both WT strains, highlighting the importance to take differences in cell line models into account when investigating bacterial host pathogen interactions. Western Blot analysis of Caco-2 cells infected with WT *C. jejuni* 11168H confirmed correlation between elevated hBD-2 and -3 mRNA with protein levels (Figure 3.2). An additional important finding was the presence of hBD-2 peptide in infected cell supernatants, while hBD-3 was mainly extracted from the cell lysate. It is tempting to speculate that these findings indicate compartmentalization of defensin expression (secretory *versus* intracellular) providing several levels of host defence. Specifically, given the suggested secretory nature of hBD-2, this peptide might target extra cellular, adhering pathogens, while hBD-3 may attack bacteria that have managed to invade the host cell. Additionally, hBD-3 might be released upon apoptosis of IEC or during the inflammatory process providing an additional extracellular line of defence.

Utilizing luciferase reporter gene assays, we aimed to investigate the transcriptional regulation of innate defence genes in response to *C. jejuni*. In addition to promoter constructs for IL-8, hBD-2, -3 we also investigated transcriptional regulation of NF-κB. The transcription factor is well established as a central player in regulating innate gene transcription and has been implicated in *C. jejuni* mediated host defence (Fox *et al.*,

2004; Hu and Hickey, 2005). For all innate genes tested, we observed a two to three fold induction in response to both WT strains 11168H and 81-176 (Figure 3.4). Compared to other stimuli, such as IL-1β, this could be considered as a relatively moderate induction. However, it is likely that *C. jejuni* has developed strategies to evade host recognition for example by phase variation of surface structures, and hence causes a considerably moderate immune response. Similar to our RT-PCR findings, there was a reproducible, yet not statistically significant trend of 81-176 leading to slightly higher levels of promoter activity (Figure 3.4). Findings at that stage clearly demonstrated dynamic cross talk between *C. jejuni* and the IEC leading to the induction of hBD-2 and hBD-3. However, in order for these peptides to play a role in disease pathogenesis they must exhibit antimicrobial activity against the bacterium. This could not be taken for granted as the antimicrobial activity of hBDs varies greatly according to peptide and organism tested (Pazgier *et al.*, 2006). Additionally, several mechanisms have been reported that allow bacteria to become resistant to cationic peptides (Peschel and Sahl, 2006). We therefore investigated the bactericidal activity of hBD-1, -2 and -3 against WT *C. jejuni* 11168H and found inducible hBD-2 and -3 to be highly potent and fast acting bactericidal agents against *C. jejuni*, further highlighting their potential role in *C. jejuni* disease pathogenesis (Figure 4.1). In contrast to hBD-2 and -3, bactericidal activity of hBD-1 was found to be significantly lower and more variable (Figure 4.1). However, the observed synergistic or at least additive bactericidal effects of hBD-1 with lysozyme indicated how AMPs from different families might combine forces and hence enhance their individual properties (Figure 4.6). Although the exact concentration of hBDs at the GI mucosal surface remains unknown, killing capacity of defensins in the $10^{-7}$M range observed in the present study is comparable to those reported for other Gram-negative bacteria (Lee *et al.*, 2004). Speculating about a potential role of these peptides *in-vivo*, one has to take the salt sensitive nature of defensins into consideration.

Higher salt concentrations in the GI tract, might inhibit bactericidal activity and therefore be considerably lower than in the more favorable low salt experimental conditions. However, regardless of *in-vivo* salt concentrations, given the observed fast acting bactericidal effect of hBD-2 and -3, close contact to the host cell or even intracellular presence of *C. jejuni* could allow defensin mediated killing to take place in a "microenvironment" where high peptide concentrations can be achieved and hence salt concentrations may become less relevant. Further studies are needed to support these speculations.

The exact mechanism of how defensins mediate their antimicrobial activity remains unknown, however increasing evidence suggest structural changes to the bacterial membrane leading to pore formation as one of the a major mechanisms involved (Jenssen *et al.*, 2006). We were able to demonstrate structural changes in *C. jejuni* bacterial cell membrane following incubation with recombinant hBD-3 (Figure 4.9). Using SEM we observed thinning out of membranes with evidence of pore formation and leakage of intracellular contents, suggesting that the bacterial membrane might indeed be a prime target for hBD-3 (Figure 4.9). More recently, several other mechanisms for defensin mediated bactericidal activity have been suggested including interference with mRNA, DNA or protein synthesis as well as induction of proteolytic enzymes (Sahl *et al.*, 2005; Jenssen *et al.*, 2006). It is more than likely that one or several of these mechanisms are also operative and either enhance or compliment killing of *C. jejuni* leading to the observed high potency of these peptides against the bacterium. In light of the increasing resistance of *C. jejuni* to commercial antibiotics used as first line treatment for human infections (i.e. Fluroquinolones, Macrolides) (Padungton and Kaneene, 2003; Andersen *et al.*, 2006), defensins may represent a promising candidate for future therapeutic intervention.

Since its recent discovery, *C. jejuni* CPS has been investigated intensively and evidence is accumulating that this structure might play a major role in disease pathogenesis in several ways including mediating bacterial adhesion and invasion (Bacon *et al.*, 2001), serum resistance and last but not least by undergoing phase variation (Karlyshev *et al.*, 2005b). The latter mechanisms, causing constant variation/change of surface structures might allow *C. jejuni* to avoid recognition and evade host defence. Using a capsule deficient isogenic mutant strain of *C. jejuni* 11168H, *kpsM*, we investigated the potential role of CPS in modulating intestinal innate host defence. Interestingly, we were unable to detect any significant differences in innate host defence gene expression including hBD-1, -2, -3 and IL-8 (Figure 3.3) and transcriptional regulation (Figure 3.4) between mutant and parental WT strain, suggesting that the presence of this structure does not seem to be required for the host to recognize the presence of *C. jejuni* and mount innate immune defence. However, despite its limited role in host cell recognition, we hypothesized that the capsule might provide protection for *C. jejuni* against the bactericidal activity of defensins as this has been shown to be the case for other Gram-negative bacteria (Campos *et al.*, 2004). In order to address this question, we performed bactericidal assays, comparing susceptibility of *kpsM* and parental WT strain towards hBDs. Surprisingly, we did not observe a significant difference in killing activity for either hBD-2 or hBD-3 suggesting that CPS does not protect *C. jejuni* against these peptides (Figure 4.7). However, a statistically not significant but yet reproducible trend of *kpsM* mutant to be more susceptible to hBD-1 and lysozyme was noted (Figure 4.7). If true then this data suggests that the capsule provides added resistance of constitutively expressed AMPs, a property that may assist the bacterium in overcoming the initial line of host defence and hence allow adhesion and invasion to occur *in-vivo* (Figure 4.7).

Prior to the induction of host innate defence in response to microbes, it is essential for the host to sense the presence of a potential pathogen. An ancient, universal strategy host microbial sensing is the interaction of PAMPs with corresponding host PRRs (Akira, 2006; Franchi *et al.*, 2006). In the GI tract the constant exposure to the diverse commensal flora causes selective pathogen recognition to be of paramount importance. In order to tolerate the presence of commensal bacteria while ensuring protection against pathogens the GI epithelium as the frontline of defence has developed several mechanisms. Expression of luminal surface PRRs is reduced (i.e. TLR4) (Cario and Podolsky, 2000; Abreu *et al.*, 2001) and/or mainly restricted to the basoloateral surface (i.e. TLR5) (Gewirtz *et al.*, 2001a). Additionally, signaling of TLRs is tightly regulated for example *via* co-expression of the TLR-signaling suppressor Tollip maintaining relative hyporesponsiveness to luminal bacteria and their products (Melmed *et al.*, 2003).

Recent studies investigating host sensing of *C. jejuni via* TLR5 demonstrated that unlike *Salmonella* which engages host TLR5 (Gewirtz *et al.*, 2001a), *C. jejuni* flagellin was found to have weak immune-stimulatory properties towards IEC TLR5 (Andersen-Nissen *et al.*, 2005; Watson and Galan, 2005; Johanesen and Dwinell, 2006). The information that bacterial invasion is crucial for IEC immunity, coupled with minimal involvement of TLR5 led us to hypothesize that the intracellularly located NOD family could be likely PRR(s) for *C. jejuni*.

Firstly, we confirmed the association between the intracellular presence of *C. jejuni* (or at least *C. jejuni* components) and induction of innate host defence by showing that infection of Caco-2 cells with PFA-fixed bacteria led to a significantly reduced induction of IL-8 and hBD-2 (Figure 5.1). Immuno-staining of *C. jejuni* in Caco-2 co-cultures was performed to demonstrate that inactivated bacteria were indeed located mainly on the extracellular surface, while a significant proportion of live bacteria

invaded the host cell within the 5h infection period (Figure 5.3). These findings further supported our hypothesis that bacterial sensing might take place intracellularly. In recent years, NOD-like receptors have emerged as family of cytoplasmic receptors, responsible for the sensing PGN of Gram-positive and Gram-negative bacteria ultimately leading to activation of NF-κB and transcription of several innate defence genes including IL-8 and hBD-2 (Inohara *et al.*, 2002; Philpott and Girardin, 2004; Franchi *et al.*, 2006; Fritz *et al.*, 2006; Strober *et al.*, 2006). Two members of the NOD receptor family found to be expressed in the GI tract are NOD1 and NOD2. We investigated expression of both receptors in our cell line model during *C. jejuni* infection. NOD1 was found to be expressed constitutively both in Caco-2 and HT-29 cells, with no further modulation of mRNA levels occurring over a 24h infection period with WT *C. jejuni* strain 11168H and 81-176 (Figure 5.4). In contrast, we were unable to detect expression of NOD2 in unstimulated IEC and mRNA levels remained absent during *C. jejuni* infection regardless of WT strain tested or cell line utilized (Figure 5.4 and 5.5). In the same experiments, we confirmed appropriate infectivity by following expression of hBD-2, a peptide whose induction has been shown to depend on activation of NOD1 (Boughan *et al.*, 2006). Induction of hBD2 in the absence of NOD2 further highlighted NOD1 as a potential intracellular sensor for *C. jejuni* (Figure 5.4 and 5.5). Si RNA, specifically targeting NOD1 (siNOD1) allowed us to reduce expression of this receptor in our model cell line system and we were able to demonstrate a significant decrease in *C. jejuni* mediated IL-8 and hBD-2 expression in siRNA treated cells (Figure 5.6). Significant reduction was confirmed with two *C. jejuni* WT strains on IL-8 and hBD-2 (Figure 5.6), transcriptional activity (Figure 5.7 and 5.9) and also the IL-8 protein level (Figure 5.8) unequivocally highlighting IEC NOD1 as a major PRR for mediating host innate defence against *C. jejuni*. However, although marked knock-down of the NOD1 gene was observed (Figure 5.6) its corresponding inhibitory effect

on IL-8 and hBD2 gene and promoter function (Figure 5.6, 5.7 and 5.9) ranged between 40 and 60%, suggesting NOD1-independent signaling events are also involved in IEC innate immune gene regulation. Several studies have implicated MAP kinase pathways in *C. jejuni*-mediated IL-8 production (MacCallum *et al.*, 2005; Watson and Galan, 2005) and it is most likely that they are also operative in our model system.

Despite undetectable levels of NOD2 in Caco-2 cells, expression can be induced under inflammatory conditions *in-vivo*, which led us to investigate the potential role for this PRR in epithelial sensing of *C. jejuni*. Interestingly, NOD2 did not enhance *C. jejuni* mediated IL-8 or hBD-2 (Figure 5.10 and 5.11). Importantly, functionality of NOD2 was confirmed by using *S. pneumoniae*, known to activate the receptor, suggesting that NOD2 plays a minor role in sensing *C. jejuni* in IEC (Figure 5.11). However, this might not be the case in other innate immune cells such as DCs, which express NOD2 and differ in their ability to uptake *C. jejuni* (Ogura et al., 2001). Unlike IECs, DCs are phagocytic in nature and therefore may lead to differential immune responses.

Taken together, we demonstrated NOD1 and not NOD2 involvement in sensing the intracellular presence of *C. jejuni* leading to the induction of IL-8 and hBD-2. Reduced expression of NOD1 should lead to an impaired antimicrobial host response (including reduced hBD-2) allowing for increased bacterial adhesion and invasion to occur. In order to investigate this hypothesis we performed invasion assays on cells transfected with either siNOD1, siNEG, NOD2 overexpression plasmid and untreated control cells. Following 20h incubation time, allowing for bacterial invasion and activation of innate defence including secretion of hBD-2 to occur, the number of live intracellular *C. jejuni* 81-176 organisms was evaluated using a gentamicin protection assay. Bacterial invasion was observed in untreated control Caco-2 cells and numbers obtained were similar to those from cells treated with negative control siRNA (siNEG) as well as NOD2 expressing cells. Importantly, we were able to demonstrate a significant increase in live

intracellular *C. jejuni* in cells treated with either of the siRNA sequences specific for NOD1 (Figure 5.12) suggesting that NOD1-mediated cellular events do contribute to the number of intracellular bacteria. Whether this is due to increased invasion or survival or due to decrease in bacterial transcytosis remains a question for future work (McKay *et al.*, 2007).

There are major limitations to the use of cell lines as *in-vitro* models, which include the absence of several cell types (e.g. Paneth cells, goblet cells) and structural organization (villi and crypts) of the GI mucosa *in-vivo*. Further, *C. jejuni* is a commensal in most wild and domestic animals; hence studies of disease pathogenesis in animal models to date have been limited. In order to establish an improved model of *C. jejuni* infection addressing some of these limitations, we used intestinal forceps biopsies to study host-*C. jejuni* interactions. Following optimization allowing the *in-vitro* culture of tissue samples of small and large bowel for up to 12h, co-culture experiments with WT *C. jejuni* were preformed and samples examined for the presence of the bacterium at the epithelial surface. The major differences to cell lines became immediately evident, when most *C. jejuni* were found to be trapped within the thick mucus layer covering the IEC (Figure 6.3). To date only few studies have reported on the interaction of *C. jejuni* with human intestinal biopsies in an *ex-vivo* model. Similar to our findings, a study by Grant and colleagues also demonstrated the association of *C. jejuni* in large clusters within the mucus overlaying the epithelium of duodenal tissue samples (Grant *et al.*, 2006). Although the majority of bacteria were found in the mucus layer of the small bowel, it was particularly in those areas where we observed close contact of *C. jejuni* with the intestinal epithelium possibly representing true adhesion (Figure 6.3). It is tempting to speculate that *C. jejuni* might use the potentially protected environment of the mucus to multiply prior to attacking the epithelium layer. The suggested chemotactic properties of mucus towards *C. jejuni* lend further support for this

hypothesis (Hendrixson *et al.*, 2001). In comparison to findings in the small bowel (i.e. terminal ileum), we were unable to detect close bacterial contact to the epithelium layer in the colon, the reason for which remains unknown. One possible explanation might be that culture conditions were suboptimal for *C. jejuni* and in favour for the eukaryotic cell. With *C. jejuni* requiring microaerophilic conditions for ideal growth, relatively high oxygen levels might prevent the bacterium from exhibiting its full virulence and hence mask important events occurring *in-vivo*. In order to address this problem and further optimize existing *in-vitro* models, we utilised a vertical diffusion chamber system attempting to provide ideal growth conditions for both *C. jejuni* and Caco-2 cells. Preliminary results provided promising data, suggesting that *C. jejuni* exhibits greater virulence when exposed to microaerophilic conditions (Figure 6.7a and b). However, further experiments are needed to confirm and extend these data.

In future work, we aim to combine both models by placing intestinal biopsies in the vertical diffusion chamber system, creating a close to *in-vivo* setting. This would open exciting opportunities to study *C. jejuni* disease pathogenesis in health and disease. For example, access to tissue from children suffering from chronic inflammatory bowel diseases (i.e. Crohn's Disease and Ulcerative Colitis), known to have defects in their innate defence set up (i.e. impaired defensins expression, NOD mutations) will add further to the strength of this novel system.

In conclusion, this study highlights the important role of intestinal epithelial innate host defence in *C. jejuni* disease pathogenesis. We identified hBD-2 and hBD-3 as AMPs that are not only induced during *C. jejuni* infection but also exhibit a highly potent and fast acting bactericidal activity against the bacterium. They are therefore likely to play a major role in disease pathogenesis possibly *via* enhanced bacterial clearance in the healthy host. Furthermore, with the increase of antibiotic resistant *C. jejuni* strains, hBDs represent promising candidates for developing novel treatment strategies in the future. With the identification of IEC NOD1 as the first PRR involved in the sensing of *C. jejuni* and mediating host bactericidal response, several new questions arise that can now be investigated, such as the role of bacterial invasion and or intracellular processing of *C. jejuni* PGN leading to activation of NOD1.

However, despite the discovery of these important host defence mechanisms *C. jejuni* causes disease in otherwise healthy individuals. It is therefore likely that the bacterium has evolved several mechanisms allowing evasion of initial host recognition leading to the observed spectrum of disease. Likely mechanisms to be involved include the *N*- and *O*-linked glycosylation system, phase variability of surface structures such as CPS and LOS as well as changes in the flagellin locus avoiding stimulation of TLR5. Further studies are needed to explore both bacterial and host mechanisms in order to complete our understanding of *C. jejuni* disease pathogenesis. The development of novel *ex-vivo* systems to study *C. jejuni* disease pathogenesis has therefore great potential to provide us with previously unavailable opportunities to unravel some of the secrets behind this major human pathogen.

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# A major role for intestinal epithelial nucleotide oligomerization domain 1 (NOD1) in eliciting host bactericidal immune responses to *Campylobacter jejuni*

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# Intestinal Innate Immunity to *Campylobacter jejuni* Results in Induction of Bactericidal Human Beta-Defensins 2 and 3

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# Innate immune defence in the human gastrointestinal tract

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