Accepted Manuscript

Title: Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut

Authors: Kim Van Deun, Frank Pasmans, Richard Ducatelle, Bram Flahou, Kris Vissenberg, An Martel, Wim Van den Broeck, Filip Van Immerseel, Freddy Haesebrouck

PII: S0378-1135(07)00576-7
DOI: doi:10.1016/j.vetmic.2007.11.027
Reference: VETMIC 3897

To appear in: *VETMIC*

Received date: 22-8-2007
Revised date: 15-11-2007
Accepted date: 21-11-2007

Please cite this article as: Van Deun, K., Pasmans, F., Ducatelle, R., Flahou, B., Vissenberg, K., Martel, A., Van den Broeck, W., Van Immerseel, F., Haesebrouck, F., Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.11.027

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut

Running title: Mechanism of *Campylobacter jejuni* colonization in poultry

Kim Van Deun^{a,*o}, Frank Pasmans^{ao}, Richard Ducatelle^a, Bram Flahou^a, Kris Vissenberg^b, An Martel^a, Wim Van den Broeck^c, Filip Van Immerseel^a, Freddy Haesebrouck^a

^a Department of Pathology, Bacteriology and Avian Diseases, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium. ^b Biology Department, Plant Physiology and Morphology, Groenenborgerlaan 171, B-2020 Antwerp University, Belgium. ^c Department of Morphology, Ghent University, Salisburylaan 133, 9820 Merelbeke.

*Corresponding author: Van Deun Kim; Mailing address: Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium;

Phone: ++32/9 264 73 76

Fax: ++32/9264 74 94

E-mail: kim.vandeun@ugent.be

^o Equally contributed to this study

21 **Abstract**

22 Although poultry meat is now recognized as the main source of *C. jejuni* gastroenteritis, little
 23 is known about the strategy used by the bacterium to colonize the chicken intestinal tract. In
 24 this study, the mechanism of *C. jejuni* colonization in chickens was studied using 4 human
 25 and 4 poultry isolates of *C. jejuni*. The *C. jejuni* strains were able to invade chicken primary
 26 cecal epithelial crypt cells in a predominantly microtubule dependent way (5/8 strains).
 27 Invasion of cecal epithelial cells was not accompanied by necrosis or apoptosis in the cell
 28 cultures, nor by intestinal inflammation in a cecal loop model. *C. jejuni* from human origin
 29 displayed a similar invasive profile compared to the poultry isolates. Invasiveness of the
 30 strains *in vitro* correlated with the magnitude of spleen colonization in *C. jejuni* inoculated
 31 chicks. The *C. jejuni* bacteria that invaded the epithelial cells were not able to proliferate
 32 intracellularly, but quickly evaded from the cells. In contrast, the *C. jejuni* strains were
 33 capable of replication in chicken intestinal mucus. These findings suggest a novel
 34 colonization mechanism by escaping rapid mucosal clearance through short term epithelial
 35 invasion and evasion, combined with fast replication in the mucus.

36

37 **Keywords:** *Campylobacter jejuni*, poultry, invasion, colonization

38

39 **1. Introduction**

40

41 Food-borne gastroenteritis is frequently associated with the handling and consumption of
 42 *Campylobacter jejuni* contaminated food products such as meat, raw milk (Butzle and
 43 Oosterom, 1991) and unchlorinated water (Skirrow, 1991). *C. jejuni* is widespread in animals,
 44 including dogs (Lee *et al.*, 2004), but as far as the source for human enteric disease is
 45 concerned, infected poultry meat and cross-contamination of food pose the main threat

(Wingstrand *et al.*, 2006). Although severe complications may arise from infection with *C. jejuni* such as the Guillain-Barré syndrome (Hughes and Cornblath, 2005), the disease in humans usually presents as a self-limiting enteritis. *C. jejuni* may belong to the normal avian microbiota and despite a huge colonization number of up to 10^8 colony forming units (cfu) per gram intestinal content, chickens generally remain asymptomatic (Dhillon *et al.*, 2006). In contrast with the hypothesis that *C. jejuni* is a mere commensal bacterium in chickens, some studies report the ability of *C. jejuni* to invade the chicken intestinal mucosa (Knudsen *et al.*, 2006) and cause systemic infection (Sanyal *et al.*, 1984).

C. jejuni usually appears in broiler flocks at an age of two to three weeks (Gregory *et al.*, 1997), which coincides with a drop in maternal antibody titers (Sahin *et al.*, 2001). It has been reported that as few as 2 to 35 cfu (Stern *et al.*, 1988; Knudsen *et al.*, 2006) are sufficient for cecal colonization, while others mention a higher minimal inoculation dose, up to 5×10^4 cfu for 14 day old chicks (Ringoir *et al.*, 2007). The ability to colonize the chicken gut varies between strains (Young *et al.*, 1999), but once one chicken is infected, the bacterium spreads rapidly through the whole flock, resulting in infection of almost 100% of the chickens (Lindblom *et al.*, 1986). Several genes, including the *dnaJ*, *pldA* and *cadF* genes, have been associated with colonization of the intestinal tract (Zirpin *et al.*, 2001). It has been reported that intact motile flagella are necessary for efficient colonization (Nachamkin *et al.*, 1993). Wassenaar *et al.*, however, concluded that it is the presence of flagellin A rather than motility that contributes to colonization (1993). *Campylobacter* invasion antigen (Cia) proteins also play a role in chicken gut colonization (Biswas *et al.*, 2007). These proteins are secreted in the presence of chicken serum and mucus. It was the purpose of this study to examine the colonization strategy of *C. jejuni* in chickens. Therefore the interaction of the bacterium with chicken primary cecal epithelial cells was studied *in vitro*, while inflammation was examined

using a cecal loop model. Finally, colonization was evaluated *in vivo* by inoculating day of hatch chicks.

2. Materials and Methods

2.1. Experimental animals

Specific pathogen free White Leghorn Chickens (Charles River Laboratories, Brussels, Belgium) and commercial Leghorns were kept in brooder batteries. Food and water were provided *ad libitum*. Husbandry, experimental procedures, euthanasia methods and biosafety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University. Prior to use, chicks were examined weekly for the presence of *Campylobacter* and *Salmonella* in the feces. This was done by enrichment of the collected feces in buffered peptone water (BPW, Oxoid, Basingstroke, England) for 24 hours, after which the suspension was plated on Modified *Campylobacter* Charcoal Differential Agar (mCCDA, Oxoid) or Brilliant Green Agar (BGA, LabM Limited, Lancashire, England) plates for the detection of *C. jejuni* or *Salmonella* respectively.

2.2. Bacterial strains and growth conditions

Campylobacter jejuni strains KC 40, KC 51, KC 69.1 and KC 96.1 from poultry origin (kindly provided by Dr. Marc Heyndrickx, ILVO, Melle, Belgium) and R-27450, R-27456, R-27461 and R-27473 (kindly provided by Prof. Dr. Peter Vandamme, Ghent University, Ghent, Belgium) isolated from human patients with gastroenteritis, were used in the *in vitro* invasion assay. Based on experimental results described further in this paper, strains KC 40, R-27456

and R-27473, which displayed a high, medium and low invasion profile in chicken primary cecal epithelial cells from crypts, were selected for further experiments. All strains were routinely cultured in Nutrient Broth No. 2 (Oxoid), supplemented with *Campylobacter* specific growth supplements (SR117 and SR0232, Oxoid) at 42°C under microaerophilic conditions (5% O₂, 5% CO₂, 5% H₂, 85% N₂) for 24 hours. *Salmonella* Enteritidis 76SA88 was used as positive control for invasion and inflammation assays and cultured in Luria Bertani broth (LB, Sigma, Bornem, Belgium) at 37°C for 24 hours. *Escherichia coli* DH5α was used in this study as a negative control for invasion in chicken primary cecal epithelial cells and routinely cultured on Luria Bertani agar or broth.

2.3. Cell lines

The human colon carcinoma cell line T84 was grown in 44% D-MEM, 44% F-12 (Gibco, Merelbeke, Belgium), 10% fetal calf serum (FCS) (Integro B.V., aa Dieren, Netherlands), 1% L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37°C in 5% CO₂ atmosphere.

2.4. Isolation of chicken primary cecal epithelial cells from crypts

Ceca from commercial brown laying hens at the age of 12 to 20 weeks were used to isolate primary epithelial cells from crypts according to a modified protocol of Booth *et al.*, (1995). Ceca were washed with HBSS with Ca²⁺ and Mg²⁺ to remove fecal content, diced and digested at 37°C in digestion medium (99% DMEM, 1% FCS, 25 µg/ml gentamicin (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin, 375 U/ml collagenase (Sigma) and 1 U/ml dispase (Roche, Vilvoorde, Belgium) until crypts were found floating in the medium. The

crypts were centrifuged on a sorbitol gradient (30 x g, 5 min, 37°C) and seeded in a 96 well plate at a concentration of 500 crypts per well in 200 µl cell medium (97.5% DMEM, 2.5% FCS, 10 µg/ml insulin (Sigma), 5 µg/ml transferrin (Sigma), 1.4 µg/ml hydrocortisone (Sigma) + 1 µg/ml fibronectin (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin). After 40 hours the individual crypts had spread out on the bottom of the well. Only wells with a minimal surface coverage of 70-80% were used.

2.5. Isolation of embryonal chicken fibroblasts

Ten day old chicken embryos were euthanized and head, legs and wings were removed. The abdomen was diced and washed with PBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The diced fragments were transferred in an Erlenmeyer with 50 ml PBS, 10% trypsin, 100 U/ml penicillin and 100 µg/ml streptomycin and gently stirred. Fibroblasts were harvested by adding 5 ml FCS and subsequent centrifugation of the tissue fragments at 220 x g for 10 min at room temperature. The supernatant was discarded and the cells were suspended in a phosphate buffered solution containing 137.0 mM NaCl, 2.5 mM KCl, 8.0 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1.0 mM MgCl₂, 1.0 mM CaCl₂ and 20% FCS. The cell suspension was centrifuged at 220 x g for 10 min at room temperature and the supernatant was discarded. The cells were suspended in fibroblast growth medium (90% MEM, 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% potassium, 1% L-glutamine) and seeded in a 25 cm² culture flask. When grown to full density, they were subcultured in 75 cm² culture flasks before storage at -80°C.

2.6. *Adhesion of C. jejuni to chicken primary cecal epithelial cells*

Adherence of *C. jejuni* to chicken primary cecal epithelial cells was studied using Scanning Electron Microscopy (SEM). Primary cecal epithelial crypt cells were plated on HCl treated glass coverslips in 24 well plates at a density of 2500 crypts per well. After 40 hours, cells were washed and inoculated with *C. jejuni* strain KC 40 at a multiplicity of infection (m.o.i.) of 200. At different time points, cells were gently washed once and fixed overnight in 5% paraformaldehyde, 437.0 mM NaCl, 187.0 mM HEPES, 12.5 mM CaCl₂.H₂O, 5.8% glutaraldehyde, pH 7.2. Samples were post fixed with 1% osmiumtetroxide for 2 hours at room temperature. After dehydration through a graded series of alcohol and acetone, samples were critical point dried (Balzers, Liechtenstein) and platinum sputter-coated with a JFC-1300 Auto fine coater (Japanese Electronic Optical Laboratories, Japan). Analysis was performed on a JSM-5600LV (Japanese Electronic Optical Laboratories, Japan) scanning electron microscope.

2.7. *Invasion and intracellular survival of C. jejuni in chicken primary cecal epithelial cells, chicken embryonic fibroblast cells and T84 cells*

To examine the contribution of invasion as a possible mechanism for persistent colonization, chicken primary cecal epithelial crypt cells and chicken embryonic fibroblast cells were inoculated with *Campylobacter*, *Salmonella* or *E. coli* DH5 α . The two latter served respectively as a positive and negative control for invasion. Chicken embryonic fibroblast cells were seeded in 96-well plates at a density of 10⁵ cells per well. In all invasion assays, an m.o.i. of 200 was used. Plates were centrifuged at 500 x g for 10 min at 37°C. After 3 hours incubation at 37°C in a 5% CO₂ atmosphere, cells were washed with HBSS with Ca²⁺ and

Mg²⁺ (Gibco) to remove non-invaded bacteria. Extracellular bacteria were killed by adding 100 µg/ml gentamicin for 2 h at 37°C in a 5% CO₂ atmosphere. This concentration was lethal for all strains used in this study. Gentamicin was washed away with HBSS with Ca²⁺ and Mg²⁺. To determine the number of internalized bacteria, cells were washed, lysed with 0.25% sodium deoxycholate and tenfold dilutions were plated on mCCDA, BGA or LB plates for quantification of *C. jejuni*, *Salmonella* or *E. coli* respectively.

For visual confirmation of invasion of *C. jejuni* in chicken primary cecal epithelial crypt cells, Confocal Laser Scanning Microscopy (CLSM) was used. Cells were seeded on HCl treated glass coverslips and were allowed to adhere for 40 hours. Cells were inoculated with *C. jejuni* strain KC 40 as described above. After infection, cells were fixed in 3.6% paraformaldehyde for 20 min at room temperature. Cells were permeabilized with 0.5% Triton X-100 in PBS for 40 min, blocked with 1% bovine serum albumin (BSA) for 45 min and incubated overnight with 1/50 rabbit anti-*Campylobacter* serum (Biodesign, Saco, USA) in 1% BSA at 4°C. After washing, cells were incubated with 1/200 goat anti-rabbit antibody conjugated with 488 Alexa Fluor (Invitrogen, Molecular probes, Belgium) in 1% BSA. Cell nuclei were stained with 10 µg/ml propidium iodide for 5 min. Pictures of the cells and bacteria were taken using a Nikon C1 Confocal Laser Scanning Microscope mounted on a Nikon Eclipse E600 microscope with a 60x (NA= 1.2) water immersion lens and a filter set for simultaneous visualization of propidium iodide, FITC and a transmission image.

The contribution of the cytoskeleton to invasion in primary cecal epithelial crypt cells was studied using 2 µM cytochalasin D (Sigma) as inhibitor for actin filament polymerization and 20 µM nocodazole (Sigma) for the inhibition of microtubule formation. *Salmonella* was used as a control, since its invasion is microfilament dependent but microtubule independent

(Finlay *et al.*, 1991). Inhibitors were added 1 hour prior to infection and maintained during infection. After 3 hours, wells were washed and gentamicin (100 µg/ml) without inhibitors was added for 2 hours to kill extracellular bacteria. Intracellular bacteria were quantified as described for the invasion assay.

Intracellular survival was assessed using chicken primary cecal epithelial crypt cells and T84 cells. Invasion with *C. jejuni* strains KC 40, R-27456 and R-27473 was carried out as described in the invasion assay, but after the killing of extracellular bacteria, cells were incubated at 37°C in a 5% CO₂ atmosphere in cell medium containing 50 µg/ml gentamicin, to avoid extracellular replication of bacteria. The incubation time was set at 4, 18 and 24 hours for the primary cecal epithelial crypt cells and 3, 18 and 24 hours for T84 cells. *Salmonella* served as a positive control for intracellular survival assay only in T84 cells, because preliminary test revealed that *Salmonella* caused massive damage to primary cecal epithelial crypt cells after 5 hours incubation. After incubation, intracellular bacteria were quantified as earlier.

2.8. Evasion of *C. jejuni* from primary cecal epithelial cells

Escape from the cell layer after invasion was examined using primary chicken cecal epithelial crypt cells. An invasion assay with *C. jejuni* strains KC 40, R-27456 and R-27473 was carried out as described above. After killing of extracellular bacteria with gentamicin, cells were incubated for another 19 hours at 37°C in a 5% CO₂ atmosphere in 200 µl of cell medium without antibiotics. At regular time intervals (5 min, 20 min, 35 min, 95 min, 3 hours, 6 hours, 10 hours, 12 hours and 19 hours), the medium was replaced with fresh medium. The collected

supernatant containing evaded *C. jejuni* bacteria was titrated on mCCDA plates for quantification.

2.9. Reinvasion of *C. jejuni* from primary cecal epithelial cells

In the intracellular survival assay, no viable bacteria could be recovered after 4 hours incubation with 50 µg/ml gentamicin. Hence we concluded that after 4 hours, all bacteria had escaped the cell layer and were killed due to exposure to gentamicin. To investigate whether these escaped bacteria could reinvade the cell layer, cells were inoculated as described above, but after 2 hours of exposure to 100 µg/ml gentamicin, cells were washed and incubated in medium without gentamicin for 3 hours, thus allowing bacteria to freely escape the cell layer. After these 3 hours, extracellular bacteria, which had failed to re-invade the cell layer, were killed by addition of 100 µg/ml gentamicin for 1 hour. Re-invaded bacteria were quantified as described for the invasion assay.

2.10. Apoptosis / necrosis assay

To determine whether induction of apoptosis or necrosis in epithelial cells by *C. jejuni* could be a mechanism by which the bacterium escaped the cell layer, chicken primary cecal epithelial cells were seeded in 96-well plates and allowed to adhere for 40 hours. The cell layer was infected with *C. jejuni* at an m.o.i. of 200 in the absence of antibiotics. After 24 hours, half of the medium was replaced by fresh medium containing 10 µg/ml propidium iodide and 10 µg/ml Hoechst 33342 (Sigma). After 15 min, 3.6% paraformaldehyde was added to the medium at a final concentration of 1.8% and cells were incubated for 20 min at room temperature. After the first fixation step, all medium was gently removed while care

was taken not to wash away detaching cells and 3.6% paraformaldehyde was added for another 20 min. After fixation, paraformaldehyde was replaced by PBS. Wells were examined through a Leica DM LB2 microscope. For pictures, cells were seeded on hydrogen chloride treated glass coverslips and allowed to adhere. Fluorescence pictures of propidium iodide and Hoechst stainings were obtained using a Zeiss Axioskope microscope equipped with a Nikon DXM1200 digital camera and the appropriate filter sets.

2.11. Growth of *C. jejuni* in chicken intestinal mucus and intestinal contents

To assess the survival and replication capacity of *C. jejuni* in chicken intestinal mucus, the small intestine of commercial 20 weeks old brown laying hens was collected and gently rinsed with PBS to remove fecal material. The mucus was scraped from the mucosa with a scalpel, diluted 1/3 with HEPES (N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid, 25 mM; pH 7.4) and vortexed. The solution was centrifuged three times at 1000 x g for 10 min at 4°C. The mucus was filter sterilized by passage through a 0.45 µm pore size filter (IWAKI, International Medical, Brussels, Belgium) and stored at -80°C. Five ml phosphate buffered saline (PBS) supplemented with 5 mg/ml mucus protein was inoculated with 1×10^7 cfu/ml of strain KC 40, R-27456 or R-27473. The controls consisted of PBS, BHI and BHI supplemented with 5 mg/ml bovine serum albumin (BSA). At different time points, bacterial counts were made by titration on mCCDA.

To examine growth of *C. jejuni* in intestinal contents, the intestines of two weeks old *C. jejuni* free chickens were used. Intestinal contents were collected and 1:1 diluted with PBS. *C. jejuni* was incubated in autoclaved and non-autoclaved intestinal material under microaerophilic conditions at 42°C and growth was assessed after 24 hours by titration as described above.

2.12. Colonization of day of hatch chickens with *C. jejuni* strains

To determine whether the variation in invasive capacity of *C. jejuni in vitro* is translated in a different colonization profile *in vivo*, day of hatch brown layer type chicks, free from *Campylobacter*, were orally inoculated with 0.2 ml of a PBS suspension containing 1×10^8 cfu/ml of *C. jejuni* strains KC 40, R-27456 or R-27473, a highly, medium and low invasive strain, respectively. On days 1, 4, 6, 8 and 12 after inoculation, four chicks were sacrificed and the ceca, spleen and liver were removed for bacteriological analysis. Samples were diluted 1/10 in BPW homogenized and a volume of 120 μ l was titrated on mCCDA plates. After 24 hours incubation at 42 °C in microaerophilic conditions, colonies were counted. Negative samples were, after enrichment in BPW, 1/10 diluted in Nutrient Broth No. 2, supplemented with selective supplement and *Campylobacter* growth supplement SR117 and SR0232. After 24 hours incubation at 42°C in microaerophilic conditions, the suspension was plated on mCCDA.

2.13. Cecal loop model

A cecal loop model was used to examine inflammation during infection of chicks with *C. jejuni* strains KC 40, R-27456 and R-27473, while *Salmonella* was used as positive control for intestinal inflammation. One day old commercial white Leghorn chicks were kept in a *Campylobacter* and *Salmonella* free environment and screened once a week for the presence of *Campylobacter* and *Salmonella* in their feces. At the age of three weeks, chicks were used for the experiment. Twelve hours before surgery, buprenorfine was administered. Chicks were anaesthetized with isofluran and the abdominal cavity was opened with an incision of 2 inch, caudal of the sternum. The ceca were exposed and one loop in each cecum was constructed

using Vicryl 4/0 surgical suture. Loops were injected with 0.5 ml of a 1×10^8 cfu/ml *Campylobacter* or *Salmonella* containing suspension in PBS, while the second cecum served as negative control and was injected with 0.5 ml PBS. After injection, the cecum was repositioned in the abdominal cavity and the peritoneum, muscles and skin were sutured. After 24 hours the chickens were euthanized by intravenous injection with T61 and ceca were fixed overnight in neutral buffered 10% formaldehyde at room temperature. Samples of spleen and liver were taken for bacteriological analysis.

For histological examination, ceca were embedded in paraffin and sections of 3 μ m thickness were cut and hematoxylin and eosin (HE) stained. Sections were examined using a Leica DM LB2 microscope. Pictures were taken using Leica DFC 320 camera and Leica IM50 imaging software.

2.14. Statistical analysis

Differences in invasion and growth were tested by means of two sided Student *t* test. Differences between strains were analyzed with ANOVA. For colonization studies, a Kruskal-Wallis test was performed. All bacteriological results were $\log_{10}$ transformed to obtain normally distributed data.

3. Results

3.1. *C. jejuni* is able to adhere to and invade in chicken primary cecal epithelial cells

Adhesion on crypt cells was tested *in vitro* using Scanning Electron Microscopy (SEM). SEM revealed focal adherence of bacteria to the crypt cell surface. Bacteria were locally clustered

instead of evenly distributed. Redistribution and aggregation of microvilli was only seen at the sites of bacterial adherence (Fig.1).

To investigate whether invasion occurred, a gentamicin protection assay was used on the isolated crypt cells. At a minimal threshold for invasion of $\log_{10}$ 1.0 cfu/ml, all strains were able to invade the primary cecal epithelial cells, but there was a strain dependent variation in the ability to do so ($P < 0.001$): Strain KC 40 was more invasive than all other strains tested ($P < 0.01$), while both KC 69.1 and R-27461 were more invasive than R-27461 ($P < 0.05$). Of the 8 strains tested, 6 strains had an invasive capacity between $\log_{10}$ 2.0 and $\log_{10}$ 3.0 cfu/ml, strain KC 40 being the most invasive of the strains used in this study ($\log_{10}$ 3.4 ± 0.1 cfu/ml). *Salmonella* served as a positive control and invaded the cell layer at $\log_{10}$ 5.2 ± 0.1 cfu/ml, while *E. coli* DH5 α , which served as a negative control could not be recovered. Results are summarized in table 1. *C. jejuni* and *Salmonella* invaded the primary cecal epithelial cells to a significant lesser extent than T84 cells (data not shown). In contrast with primary cecal epithelial cells and *Salmonella*, *C. jejuni* was not able to invade chicken embryonic fibroblast cells (data not shown).

Because of the low number of invasive bacteria, confocal laser scanning microscopy was used to confirm invasion. Intracellular bacteria were revealed microscopically using immunofluorescence (Fig. 2). Single confocal sections at 0.3 μ m, 0.9 μ m, 1.2 μ m and 2.4 μ m away from the bottom of the cells were made, revealing intracellular bacteria evenly distributed throughout the cytoplasm.

3.2. Invasion in chicken primary cecal epithelial cells is predominantly microtubule dependent

Since redistribution of microvilli was observed using SEM, contribution of cytoskeletal rearrangement during the invasion of cecal epithelial cells was examined. Invasion of 5 from

8 *C. jejuni* strains was inhibited by 20 μ M nocodazole. Remarkably, treatment with 2 μ M cytochalasin D increased invasion for two strains. Poultry strain KC 40 was inhibited by both nocodazole and cytochalasin, while invasion of strains KC 51 and R-27473 was not influenced by any of the inhibitors. Results are summarized in table 1.

3.3. *C. jejuni* is not able to survive intracellularly in chicken primary cecal epithelial cells

Invasion of *C. jejuni* in chicken cecal epithelial cells could play a role in its persistent colonization. We therefore examined the survival capacity in chicken primary epithelial cecal cells. No surviving bacteria could be recovered from 4 hours after gentamicin incubation onwards. In T84 cells, viability of *C. jejuni* was reduced after prolonged infection and in case of strain R-27473, no viable bacteria could be recovered after 24 hours. This was in sharp contrast with *Salmonella*: the prolonged infection did not alter the number of viable intracellular bacteria (mean Log 6.3 ± 0.1). Data is summarized in fig 3.

3.4. *C. jejuni* can evade and reinvade chicken primary cecal epithelial cells without inducing cytotoxicity

Shortly after invasion of crypt cells, *Campylobacter* was seen in the medium. Release of bacteria appeared to be most pronounced during the first five minutes and dropped remarkably afterwards to less than 5 cfu ml⁻¹ min⁻¹ (Fig. 4). Propidium iodide exclusion and the absence of condensed chromatin by Hoechst 33342 staining revealed that no necrosis or apoptosis was caused within 24 hours after infection in the absence of antibiotics (data not shown). To examine whether there was an invasion - evasion cycle, bacteria were allowed to re-invade the cells for 3 hours, as described in the Materials and Methods section. Compared

to the number of intracellular bacteria as determined in the invasion assay, 65% of strain R-27456 ($\log_{10} 2.7 \pm 0.3$ cfu/ml) and 38% of strain R-27473 ($\log_{10} 1.7 \pm 0.2$ cfu/ml) could be recovered, whereas KC 40 was not.

3.5. *C. jejuni* is able to survive and multiply in chicken intestinal mucus but not in intestinal contents

Since *C. jejuni* did not seem to survive intracellularly, its ability to multiply in chicken intestinal mucus or cecal content was examined. No difference in growth was detected between PBS supplemented with 5 mg/ml isolated chicken mucus (average $\log_{10} 9.0 \pm 0.3$ cfu/ml) and BHI controls (average $\log_{10} 9.1 \pm 0.3$ cfu/ml; $P > 0.05$) inoculated with $\log_{10} 6$ cfu/ml after 24 hours in a microaerophilic atmosphere at 42°C. Negative controls consisted of *C. jejuni* growth in PBS and PBS supplemented with 5 mg/ml BSA. All strains were killed in PBS alone and only strain R-27456 survived in PBS supplemented with BSA. No *Campylobacter* growth could be detected when inoculated in cecal content with $\log_{10} 6.0$ cfu/ml (average $\log_{10} 5.0 \pm 0.5$ cfu/g, $P > 0.05$) after 24 hours in a microaerophilic atmosphere at 42°C.

3.6. Invasion of chicken primary epithelial cecal cells by *C. jejuni* is not correlated with cecal colonization and inflammation in chickens, but does correlate with spleen colonization

Since variation was noticed between strains in their capacity to invade the primary epithelial cells, it was investigated whether this was reflected in the level of cecal colonization of *C. jejuni* infected chickens. The colonization of the ceca remained stable during the infection period with an average of $\log_{10} 8.6 \pm 0.1$ cfu/g cecal contents throughout the whole

experiment. No difference between the strains in their ability to colonize the cecum was observed. The detection limit of the bacteriological analysis was set at $\log_{10}$ 1.9 cfu/g and revealed the presence of *Campylobacter* in liver and spleen after enrichment in some, but not in all chickens. The highly invasive strain KC 40 was recovered from more spleen ($P < 0.05$) samples than the less invasive strains R-27456 and R-27473 (Table 2). A cecum loop model was applied to examine pathology during *C. jejuni* infection. Cecal mucosa in the *C. jejuni* infected chicks displayed normal morphology similar to that of the control birds. In the positive *Salmonella* challenged control, caseous plugs containing necrotic cells and blood were found. Foci displaying vast destruction of the structural integrity of the epithelium together with inflammatory cell infiltration were observed (Fig. 5).

4. Discussion

To investigate the colonization mechanism of *C. jejuni* in poultry, we preferred to use chicken primary cecal epithelial cells for our experiments, in order to simulate the chicken gut as close as possible and thus eliminating cell type variation, as is often the case when using cell lines from other species. Upon infection of these cecal epithelial cell layers, SEM revealed adherent bacteria which were locally clustered rather than evenly distributed on the cell surface. This might indicate that the receptors used by the bacterium to adhere, are not evenly distributed on the host cell surface. Microvilli associated with adherent bacteria appeared aggregated, suggesting a remodelling of cytoskeleton components triggered upon docking of the bacterium with the cell surface. Indeed, microtubules were necessary for the internalization process for 5 out of the 8 strains used in this study. There has been much debate about the role of actin or microtubuli for the entry of *C. jejuni* in host cells. While some report an exclusively actin dependent entry of *C. jejuni* (DeMelo *et al.*, 1989; Konkel *et al.*, 1989),

others observed both an actin and microtubuli dependent invasion (Biswas *et al.*, 2001) or no dependence at all (Russell *et al.*, 1994). However, the microtubule dependent invasion has gained importance (Oelschlaeger *et al.*, 1993; Hu *et al.*, 1999). Our findings partly reflect these different opinions. Of the 8 strains tested, 5 strains displayed a microtubule dependent pathway. Interestingly, invasion of strain KC 40 appeared to be microtubule and actin dependent, while strains KC 51 and R-27473 appeared to be unaffected by both inhibitors. Cytochalasin stimulated the uptake of 2 strains. Indeed, Wells and co-workers reported that cytochalasin can increase invasion (Wells *et al.*, 1998). It seems that the mechanism by which *C. jejuni* gains entrance inside the host cell, could be cell type and strain dependent, rather than universal.

Intracellular survival and replication of *C. jejuni* has been reported previously (Konkel *et al.*, 1992). In this study, *C. jejuni* could not survive in the primary chicken cecal epithelial cells for prolonged periods, while viable bacteria could be recovered from T84 cells after 18 hours incubation in the presence of gentamicin. Again, there was a strain dependent variation in the ability for intracellular survival in T84 cells. No replication of *C. jejuni* took place inside these T84 cells, which is in accordance with other authors who reported a gradual decline in intracellularly surviving bacteria after incubation in medium containing antibiotics, but a sustained survival if antibiotics were omitted during the prolonged survival assay (de Melo *et al.*, 1989). These observations indicate that *C. jejuni* replicates somewhere outside the epithelium and indeed, *C. jejuni* was able to replicate unhampered in mucus collected from chickens and bacterial numbers reached a plateau comparable with the BHI control. As the main site of bacterial localization (Beery *et al.*, 1988), mucus seems to provide all necessary nutrients to sustain *C. jejuni* growth. The effect of mucus on invasion remains debated. Szymanski *et al.* (1995) reported an increased binding and invasion ability in Caco-2 cells when using a viscous medium, yet McSweeney *et al.* (1987) found that the intestinal mucus

acts as barrier for *C. jejuni* adherence *in vitro* on INT 407 cells. Because *C. jejuni* was recovered from spleen and liver after experimental infection of day of hatch chickens, it was concluded that the bacterium was not hampered *in vivo* by intestinal mucus to establish close contact with the epithelial lining of the chicken gut. Moreover, invasion *in vivo* of the epithelial cells by *C. jejuni* has been observed microscopically (Welkos, 1984).

Shortly after internalization, it was observed that *C. jejuni* was released again from the cells in the medium, after which it could re-invade the cell layer and there appeared to be a strong difference between strains in their ability to do so. It is unlikely that release of the bacterium from the invaded cells is the result of cell lysis, since invasion was not accompanied by necrosis or apoptosis during a 24 hour infection period and since no significant loss of cells was observed during the evasion assay compared with the non infected control.

Our results suggest that replication inside epithelial cells is not likely to be important *in vivo* for persistent cecal colonization and that invasion alone can not be responsible for *C. jejuni*'s persistence in chickens. Rather, a dynamic process of adherence, invasion, escape from the cell layer, fast replication in mucus and re-invasion of the cell layer might explain why the bacterium is not rapidly expelled from the intestine. Interestingly, while KC 40 was the most invasive strain, it could not be recovered after the reinvasion assay. One explanation might be that the final addition of gentamicin for 1 hour was enough to kill all evading KC 40 strains due to their fast evasion rate, while the slower strains R-27456 and R-27473 remained protected intracellularly.

C. jejuni interaction with its avian host seems to be commensal in character. Indeed, in our cecal loop no gross inflammatory response, increase of heterophil influx, necrosis or tissue damage could be observed after inoculation with *C. jejuni*, in contrast with loops inoculated with *Salmonella*.

C. jejuni infection in chickens causes an increase in antibody titres (Cawthraw *et al.*, 1994), but this immune response does not result in an expected fast mucosal clearance. The inability of the secretory and systemic immune response to clear the gut from bacteria and the absence of an inflammatory response are typical aspects of the commensal microbiota (Macpherson and Uhr, 2004). In healthy individuals commensal bacteria are hindered in their translocation from the intestinal lumen to spleen and liver due to T-cell dependent immunity (Owens and Berg, 1980). The appearance of *C. jejuni* in the spleen and liver of chickens during our infection experiment shows that *C. jejuni* interaction with its avian host is not as superficial as it appears to be the case with other commensal bacteria and that its successful colonization is not solely based upon fast and efficient replication in the intestinal mucus.

The *C. jejuni* strain dependent differences in invasiveness in the chicken cecal epithelial cells were not correlated with *in vivo* colonization of the ceca of experimentally infected chickens, but rather with systemic colonization. Indeed, strain KC 40, a highly invasive strain *in vitro*, was recovered from more spleen and liver samples and in higher numbers than the medium and low invasive strains. This finding is in contrast with the observations of Hänel *et al.* (2004), who found a correlation between colonization phenotype and Caco-2 invasion, and this could be due to variation between the cells used.

Conclusion

In conclusion, our data suggest a colonization mechanism whereby *C. jejuni* is able to avoid being expelled from the chicken gut by temporal invasion and evasion of the crypt epithelial cells and persists through rapid multiplication in the mucus. This close interaction with the epithelial lining of the cecum is accompanied by translocation of the bacterium to spleen and liver, whereby invasion of spleen and liver correlates with the *in vitro* invasion capacity in chicken primary cecal cells. Despite close association with the epithelial cells of the intestine,

and subsequent internalization *in vitro*, the colonization in chickens is devoid of any inflammatory response of the chicken intestinal wall and it cannot be ruled out that *C. jejuni* is capable of manipulating the chicken immune response in order to avoid destructive inflammation of the gut, similarly to the immune modulation by some commensal bacteria.

Acknowledgements

This study was sponsored by grant R-04/002-CAMPY-Section 3 of the Belgian Federal Public Service for Health, Food Chain Safety and Environment.

K. Vissenberg is a Postdoctoral fellow of the Research Foundation Flanders (FWO). The authors acknowledge the financial support by Research Grants of the Research Foundation - Flanders (FWO). We thank Dr. Marc Heyndrickx and Prof. Dr. Peter Vandamme for providing us with the *C. jejuni* strains. The technical assistance of Bart De Pauw and Steven De Tollenaere was greatly appreciated.

References

- Beery, J., Hugdahl, M., Doyle, M., 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl. Environ. Microbiol. 54, 2365-2370.
- Biswas, D., Fernando, U., Reiman, C., Willson, P., Townsend, H., Potter, A., Allan, B., 2007. Correlation between *in vitro* secretion of virulence-associated proteins of *Campylobacter jejuni* and colonization of chickens. Curr. Microbiol. 54, 207-212.
- Biswas, D., Itoh, K., Sasakawa, C., 2003. Role of microfilaments and microtubules in the invasion of INT-407 cells by *Campylobacter jejuni*. Microbiol Immunol. 47, 469-473.
- Booth, C., Patel, S., Bennion, G., Potten, C., 1995. The isolation and culture of adult mouse colonic epithelium. Epithelial Cell Biol. 4, 76-86.

- 518 Butzler, J., Oosterom, J., 1991. *Campylobacter*: pathogenicity and significance in foods. Int.
519 J. Food Microbiol. 12, 1-8.
- 520 Cawthraw, S., Ayling, R., Nuijten, P., Wassenaar, T., Newell, D., 1994. Isotype, specificity,
521 and kinetics of systemic and mucosal antibodies to *Campylobacter jejuni* antigens,
522 including flagellin, during experimental oral infections of chickens. Avian Dis. 38, 341-
523 349.
- 524 de Melo, M., Gabbiani, G., Pechere, J., 1989. Cellular events and intracellular survival of
525 *Campylobacter jejuni* during infection of HEp-2 cells. Infect. Immun. 57, 2214-2222.
- 526 Dhillon, A., Shivaprasad, H., Schaberg, D., Wier, F., Weber, S., Bandli, D., 2006.
527 *Campylobacter jejuni* infection in broiler chickens. Avian Dis. 50, 55-58.
- 528 Finlay, B., Ruschkowski, S., Dedhar, S., 1991. Cytoskeletal rearrangements accompanying
529 *Salmonella* entry into epithelial cells. J. Cell Sci. 99, :283-296.
- 530 Gregory, E., Barnhart, H., Dreesen, D., Stern, N., Corn, J., 1997. Epidemiological study of
531 *Campylobacter* spp. in broilers: source, time of colonization, and prevalence. Avian Dis.
532 41, 890-898.
- 533 Hänel, I., Muller, J., Muller, W., Schulze, F., 2004. Correlation between invasion of Caco-2
534 eukaryotic cells and colonization ability in the chick gut in *Campylobacter jejuni*. Vet.
535 Microbiol. 101, 75-82.
- 536 Hughes, R., Cornblath, D., 2005. Guillain-Barre syndrome. Lancet 366, 1653-1666.
- 537 Hu, L., Kopecko, D.J. 1999. *Campylobacter jejuni* 81-176 associates with microtubules and
538 dynein during invasion of human intestinal cells. Infect Immun. 67,4171-4182
- 539 Knudsen, K., Bang, D., Andresen, L., Madsen, M., 2006. *Campylobacter jejuni* strains of
540 human and chicken origin are invasive in chickens after oral challenge. Avian Dis. 50, 10-
541 14.

- 542 Konkelt, M., Joens, L., 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp.
543 Infect. Immun. 57, 2984-2990.
- 544 Konkelt, M., Hayes, S., Joens, L., Cieplak, Jr. W., 1992. Characteristics of the internalization
545 and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures.
546 Microb. Pathog. 13, 357-370.
- 547 Lee, M., Billington, S., Joens, L., 2004. Potential virulence and antimicrobial susceptibility of
548 *Campylobacter jejuni* isolates from food and companion animals. Foodborne Pathog. Dis.
549 1, 223-230.
- 550 Lindblom, G., Sjorgren, E., Kaijser, B., 1986. Natural *Campylobacter* colonization in
551 chickens raised under different environmental conditions. J. Hyg. (Lond.) 96, 385-391.
- 552 Macpherson, A., Uhr, T., 2004. Induction of protective IgA by intestinal dendritic cells
553 carrying commensal bacteria. Science 303, 1662-1665.
- 554 McSweeney, E., Burr, D., Walker, R., 1987. Intestinal mucus gel and secretory antibody are
555 barriers to *Campylobacter jejuni* adherence to INT 407 cells. Infect. Immun. 55, 1431-
556 1435.
- 557 Nachamkin, I., Yang, X., Stern, N., 1993. Role of *Campylobacter jejuni* flagella as
558 colonization factors for three-day-old chicks: analysis with flagellar mutants. Appl.
559 Environ. Microbiol. 59, 1269-1273.
- 560 Oelschlaeger, T., Guerry, P., Kopecko, D., 1993. Unusual microtubule-dependent endocytosis
561 mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. Proc. Natl.
562 Acad. Sci. U. S. A. 90, 6884-6888.
- 563 Owens, W., Berg, R., 1980. Bacterial translocation from the gastrointestinal tract of athymic
564 (nu/nu) mice. Infect. Immun. 27, 461-467.

- 565 Ringoir, D., Szylo, D., Korolik, V., 2007. Comparison of 2-day-old and 14-day-old chicken
566 colonization models for *Campylobacter jejuni*. FEMS. Immunol. Med. Microbiol. 49,
567 155-158.
- 568 Russell, R.G., Blake, D.C. Jr. 1994. Cell association and invasion of Caco-2 cells by
569 *Campylobacter jejuni*. Infect Immun. 62,3773-3779.
- 570 Sahin, O., Zhang, Q., Meitzler, J., Harr, B., Morishita, T., Mohan, R. 2001. Prevalence,
571 antigenic specificity, and bactericidal activity of poultry anti-*Campylobacter* maternal
572 antibodies. Appl. Environ. Microbiol. 67, 3951-3957.
- 573 Sanyal, S., Islam, K., Neogy, P., Islam, M., Speelman, P., Huq, M., 1984. *Campylobacter*
574 *jejuni* diarrhea model in infant chickens. Infect. Immun. 43, 931-936.
- 575 Skirrow, M., 1991. Epidemiology of *Campylobacter* enteritis. Int. J. Food Microbiol. 12, 9-
576 16.
- 577 Stern, N., Bailey, J., Blankenship, L., Cox, N., McHan, F. 1988. Colonization characteristics
578 of *Campylobacter jejuni* in chick ceca. Avian Dis. 32. 330-334.
- 579 Szymanski, C., King, M., Haardt, M., Armstrong, G., 1995. *Campylobacter jejuni* motility
580 and invasion of Caco-2 cells. Infect. Immun. 63, 4295-4300.
- 581 Wassenaar, T., van der Zeijst, B., Ayling, R., Newell, D. 1993. Colonization of chicks by
582 motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A
583 expression. J. Gen. Microbiol. 139, 1171-1175.
- 584 Welkos, S., 1984. Experimental gastroenteritis in newly-hatched chicks infected with
585 *Campylobacter jejuni*. J. Med. Microbiol. 18, 233-248.
- 586 Wells, C.L., van de Westerlo, E.M., Jechorek, R.P., Haines, H.M., Erlandsen, S.L. 1998.
587 Cytochalasin-induced actin disruption of polarized enterocytes can augment
588 internalization of bacteria. Infect Immun. 66,2410-2419.

- 589 Wingstrand, A., Neimann, J., Engberg, J., Nielsen, E., Gerner-Smidt, P., Wegener, H.,
590 Molbak, K., 2006. Fresh chicken as main risk factor for campylobacteriosis, Denmark.
591 Emerg. Infect. Dis. 12, 280-285.
- 592 Young, C., Ziprin, R., Hume, M., Stanker, L., 1999. Dose response and organ invasion of
593 day-of-hatch Leghorn chicks by different isolates of *Campylobacter jejuni*. Avian Dis. 43,
594 763-767.
- 595 Ziprin, R., Young, C., Byrd, J., Stanker, L., Hume, M., Gray, S., Kim B., Konkel M., 2001.
596 Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. Avian Dis.
597 45, 549-557.

Tables

Table 1: Invasion of *C. jejuni* in primary cecal epithelial cells from chickens.

		Mean (log ₁₀ cfu/ml ± s.e.m.) of intracellular bacteria.								
		* denotes values significantly different ($P < 0.05$) from the values in the absence of the inhibitor								
Origin	Strain	Without inhibitor			With 20 µM nocodazole			With 2µM cytochalasin		
<i>C. jejuni</i> poultry	KC 40	3.4	±	0.1	2.5	±	0.2*	2.9	±	0.2*
	KC 51	2.2	±	0.2	1.7	±	0.4	2.4	±	0.4
	KC 69.1	2.6	±	0.2	0.9	±	0.3*	2.3	±	0.3
	KC 96.1	2.1	±	0.2	1.6	±	0.3	2.9	±	0.1*
<i>C. jejuni</i> human	R-27450	2.4	±	0.1	1.2	±	0.3*	2.9	±	0.2*
	R-27456	2.7	±	0.2	1.6	±	0.3*	2.8	±	0.3
	R-27461	1.9	±	0.2	0.8	±	0.2*	1.3	±	0.3
	R-27473	2.1	±	0.2	2.1	±	0.3	2.4	±	0.2
<i>Salmonella</i> Enteritidis	76SA88t	5.2	±	0.1	5.1	±	0.0	4.6	±	0.2*
<i>Escherichia coli</i>	DH5α	-			-			-		

602

Table 2: Mean ($\log_{10}$ cfu/g $\pm$ s.e.m.) of *C. jejuni* in spleen and liver after experimental infection of day of hatch chickens and number of positive samples. Chicks were euthanized on day 1, 4, 6, 8 and 12 after inoculation. Data are combined over a 12 day infection period

Strain	Spleen			Liver		
	Mean $\pm$ s.e.m.	No. of positive samples		Mean $\pm$ s.e.m.	No. of positive samples	
KC40	1.4 $\pm$ 0.2	12/20		0.8 $\pm$ 0.2	7/20	
R-27456	0.1 $\pm$ 0.1	1/20		0.2 $\pm$ 0.1	2/20	
R-27473	0.5 $\pm$ 0.1	4/17		0.2 $\pm$ 0.1	2/17	

603

604

605

Figure captions

Fig. 1. Representative scanning electron microscopy images of the surface of primary chicken cecal epithelial cells reveal adherent *C. jejuni*. (A) Surface of a non-inoculated cell showing uniform apical microvilli. (B) Adherent *C. jejuni* on the surface of primary cecal epithelial cells. The presence of bacteria coincided with clustered and distorted microvilli.

Fig. 2. Confocal Laser Scanning Microscopy image shows *C. jejuni* inside primary chicken cecal epithelial cells. Infected primary cecal epithelial cells were inoculated with strain KC 40 and fixed. Single confocal sections at (A) 0.3 μm , (B) 0.9 μm , (C) 1.2 μm and (D) 2.4 μm away from the bottom of the cells were made. Arrows and inserts show *C. jejuni* colocalizing with the propidium stained nucleus. Scale bar represents 10.0 μm .

Fig. 3. Reduction of viable intracellular *C. jejuni* bacteria in T84 cells, compared with intracellular survival in T84 of *Salmonella* Enteritidis. Number of intracellular bacteria was determined 3 hours, 18 hours and 24 hours after infection. Data represents the $\log_{10}$ mean $\pm$ s.e.m. of three independent experiments.

Fig. 4. The number of *C. jejuni* bacteria evading from chicken primary cecal epithelial cells. Bars represent the average of recovered bacteria $\pm$ s.e.m. /min after 5, 20, 35 and 95 minutes calculated from three independent experiments. KC40 is represented by black bars, R-27456 by striped bars and R-27473 by white bars.

Fig. 5. Histopathology of chicken cecal loops infected with *C. jejuni*, *Salmonella* Enteritidis or PBS for 24 hours. Sections are stained with hematoxylin and eosin. (A) Cross section of a

PBS injected cecal loop (20 x) showing healthy cecal tissue. (B) Cross section of a *C. jejuni* infected loop (20 x) showing healthy, normal cecal tissue. (C) Low power view (5 x) of *Salmonella* Enteritidis infected loop, showing necrotic lesion affecting the deeper layer of the mucosa, which is replaced by dense infiltrate of mononuclear cells lymphocytes and heterophils (arrow). A purulent caseous plug can be observed in the lumen containing necrotic cells, heterophils, blood and protein debris. Viable (a) and necrotic tissue (b) separated by an intensely colored band consisting of heterophils and monocytes (c). (D) High power view of *Salmonella* infected cecal mucosa (100 x) showing heterophil infiltration (arrowheads), haemorrhages and apoptotic/necrotic enterocytes (arrow).

1 **Tables**

2

Table 1: Invasion of *C. jejuni* in primary cecal epithelial cells from chickens.

			Mean (log ₁₀ cfu/ml ± s.e.m.) of intracellular bacteria.								
			* denotes values significantly different ($P < 0.05$) from the values in the absence of the inhibitor								
Origin	Strain		Without inhibitor			With 20 µM nocodazole			With 2µM cytochalasin		
<i>C. jejuni</i> poultry	KC 40		3.4	±	0.1	2.5	±	0.2*	2.9	±	0.2*
	KC 51		2.2	±	0.2	1.7	±	0.4	2.4	±	0.4
	KC 69.1		2.6	±	0.2	0.9	±	0.3*	2.3	±	0.3
	KC 96.1		2.1	±	0.2	1.6	±	0.3	2.9	±	0.1*
<i>C. jejuni</i> human	R-27450		2.4	±	0.1	1.2	±	0.3*	2.9	±	0.2*
	R-27456		2.7	±	0.2	1.6	±	0.3*	2.8	±	0.3
	R-27461		1.9	±	0.2	0.8	±	0.2*	1.3	±	0.3
	R-27473		2.1	±	0.2	2.1	±	0.3	2.4	±	0.2
<i>Salmonella</i> Enteritidis	76SA88t		5.2	±	0.1	5.1	±	0.0	4.6	±	0.2*
<i>Escherichia coli</i>	DH5α		-			-			-		

3

4

5

Table 2: Mean ($\log_{10}$ cfu/g $\pm$ s.e.m.) of *C. jejuni* in spleen and liver after experimental infection of day of hatch chickens and number of positive samples. Chicks were euthanized on day 1, 4, 6, 8 and 12 after inoculation. Data are combined over a 12 day infection period

Strain	Spleen			Liver		
	Mean $\pm$ s.e.m.	No. of positive samples		Mean $\pm$ s.e.m.	No. of positive samples	
KC40	1.4 $\pm$ 0.2	12/20		0.8 $\pm$ 0.2	7/20	
R-27456	0.1 $\pm$ 0.1	1/20		0.2 $\pm$ 0.1	2/20	
R-27473	0.5 $\pm$ 0.1	4/17		0.2 $\pm$ 0.1	2/17	

6

7

8

Figure 1

Figure 2

Figure 3

Figure 4

manuscript

Figure 5