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

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

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Abstract

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

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

1. Introduction

Food-borne gastroenteritis is frequently associated with the handling and consumption of *Campylobacter jejuni* contaminated food products such as meat, raw milk (Butzle and Oosterom, 1991) and unchlorinated water (Skirrow, 1991). *C. jejuni* is widespread in animals, including dogs (Lee *et al*., 2004), but as far as the source for human enteric disease is 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% O_2, 5% CO_2, 5% H_2, 85% N_2) 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% CO_2 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 Ca^{2+} and Mg^{2+} 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 Na_2HPO_4, 1.5 mM KH_2PO_4, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$ 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 $CaCl_2.H_2O$, 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* DH5α. 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 10^5 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% CO_2 atmosphere, cells were washed with HBSS with Ca^{2+} and

170 Mg^{2+} (Gibco) to remove non-invaded bacteria. Extracellular bacteria were killed by adding
171 100 µg/ml gentamicin for 2 h at 37°C in a 5% CO_2 atmosphere. This concentration was lethal
172 for all strains used in this study. Gentamicin was washed away with HBSS with Ca^{2+} and
173 Mg^{2+}. To determine the number of internalized bacteria, cells were washed, lysed with 0.25%
174 sodium deoxycholate and tenfold dilutions were plated on mCCDA, BGA or LB plates for
175 quantification of *C. jejuni*, *Salmonella* or *E. coli* respectively.
176
177 For visual confirmation of invasion of *C. jejuni* in chicken primary cecal epithelial crypt cells,
178 Confocal Laser Scanning Microscopy (CLSM) was used**.** Cells were seeded on HCl treated
179 glass coverslips and were allowed to adhere for 40 hours. Cells were inoculated with *C. jejuni*
180 strain KC 40 as described above. After infection, cells were fixed in 3.6% paraformaldehyde
181 for 20 min at room temperature. Cells were permeabilized with 0.5% Triton X-100 in PBS for
182 40 min, blocked with 1% bovine serum albumin (BSA) for 45 min and incubated overnight
183 with 1/50 rabbit anti-*Campylobacter* serum (Biodesign, Saco, USA) in 1% BSA at 4°C. After
184 washing, cells were incubated with 1/200 goat anti-rabbit antibody conjugated with 488 Alexa
185 Fluor (Invitrogen, Molecular probes, Belgium) in 1% BSA. Cell nuclei were stained with 10
186 µg/ml propidium iodide for 5 min. Pictures of the cells and bacteria were taken using a Nikon
187 C1 Confocal Laser Scanning Microscope mounted on a Nikon Eclipse E600 microscope with
188 a 60x (NA= 1.2) water immersion lens and a filter set for simultaneous visualization of
189 propidium iodide, FITC and a transmission image.
190
191 The contribution of the cytoskeleton to invasion in primary cecal epithelial crypt cells was
192 studied using 2 µM cytochalasin D (Sigma) as inhibitor for actin filament polymerization and
193 20 µM nocodazole (Sigma) for the inhibition of microtubule formation. *Salmonella* was used
194 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% CO_2 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% CO_2 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 x 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 x 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 µl 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 x 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 µm 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* DH5α, 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 µm, 0.9 µm, 1.2 µm and 2.4 µm away from the bottom of the cells were made, revealing intracellular bacteria evenly distributed throughout the cyptoplasm.

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 μM nocodazole. Remarkably, treatment with 2 μM 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 ± 0.3 cfu/ml) and 38% of strain R-27473 (log_{10} 1.7 ± 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 ± 0.3 cfu/ml) and BHI controls (average log_{10} 9.1 ± 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 ± 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 ± 0.1 cfu/g cecal contents throughout the whole

394 experiment. No difference between the strains in their ability to colonize the cecum was
395 observed. The detection limit of the bacteriological analysis was set at $\log_{10}$ 1.9 cfu/g and
396 revealed the presence of *Campylobacter* in liver and spleen after enrichment in some, but not
397 in all chickens. The highly invasive strain KC 40 was recovered from more spleen ($P < 0.05$)
398 samples than the less invasive strains R-27456 and R-27473 (Table 2). A cecum loop model
399 was applied to examine pathology during *C. jejuni* infection. Cecal mucosa in the *C. jejuni*
400 infected chicks displayed normal morphology similar to that of the control birds. In the
401 positive *Salmonella* challenged control, caseous plugs containing necrotic cells and blood
402 were found. Foci displaying vast destruction of the structural integrity of the epithelium
403 together with inflammatory cell infiltration were observed (Fig. 5).
404
405 **4. Discussion**
406
407 To investigate the colonization mechanism of *C. jejuni* in poultry, we preferred to use chicken
408 primary cecal epithelial cells for our experiments, in order to simulate the chicken gut as close
409 as possible and thus eliminating cell type variation, as is often the case when using cell lines
410 from other species. Upon infection of these cecal epithelial cell layers, SEM revealed adherent
411 bacteria which were locally clustered rather than evenly distributed on the cell surface. This
412 might indicate that the receptors used by the bacterium to adhere, are not evenly distributed
413 on the host cell surface. Microvilli associated with adherent bacteria appeared aggregated,
414 suggesting a remodelling of cytoskeleton components triggered upon docking of the
415 bacterium with the cell surface. Indeed, microtubules were necessary for the internalization
416 process for 5 out of the 8 strains used in this study. There has been much debate about the role
417 of actin or microtubuli for the entry of *C. jejuni* in host cells. While some report an
418 exclusively actin dependent entry of *C. jejuni* (DeMelo *et al.*, 1989; Konkel *et al.*, 1989),

419 others observed both an actin and microtubuli dependent invasion (Biswas *et al*., 2001) or no
420 dependence at all (Russell *et al.*, 1994). However, the microtubule dependent invasion has
421 gained importance (Oelschlaeger *et al.*, 1993; Hu *et al.*, 1999). Our findings partly reflect
422 these different opinions. Of the 8 strains tested, 5 stains displayed a microtubule dependent
423 pathway. Interestingly, invasion of strain KC 40 appeared to be microtubule and actin
424 dependent, while strains KC 51 and R-27473 appeared to be unaffected by both inhibitors.
425 Cytochalasin stimulated the uptake of 2 strains. Indeed, Wells and co-workers reported that
426 cytochalasin can increase invasion (Wells *et al.*, 1998). It seems that the mechanism by which
427 *C. jejuni* gains entrance inside the host cell, could be cell type and strain dependent, rather
428 than universal.
429 Intracellular survival and replication of *C. jejuni* has been reported previously (Konkel *et al.*,
430 1992). In this study, *C. jejuni* could not survive in the primary chicken cecal epithelial cells
431 for prolonged periods, while viable bacteria could be recovered from T84 cells after 18 hours
432 incubation in the presence of gentamicin. Again, there was a strain dependent variation in the
433 ability for intracellular survival in T84 cells. No replication of *C. jejuni* took place inside
434 these T84 cells, which is in accordance with other authors who reported a gradual decline in
435 intracellularly surviving bacteria after incubation in medium containing antibiotics, but a
436 sustained survival if antibiotics were omitted during the prolonged survival assay (de Melo *et*
437 *al.*, 1989). These observations indicate that *C. jejuni* replicates somewhere outside the
438 epithelium and indeed, *C. jejuni* was able to replicate unhampered in mucus collected from
439 chickens and bacterial numbers reached a plateau comparable with the BHI control. As the
440 main site of bacterial localization (Beery *et al.*, 1988), mucus seems to provide all necessary
441 nutrients to sustain *C. jejuni* growth. The effect of mucus on invasion remains debated.
442 Szymanski *et al*. (1995) reported an increased binding and invasion ability in Caco-2 cells
443 when using a viscous medium, yet McSweegan *et al*. (1987) found that the intestinal mucus

444 acts as barrier for *C. jejuni* adherence *in vitro* on INT 407 cells. Because *C. jejuni* was
445 recovered from spleen and liver after experimental infection of day of hatch chickens, it was
446 concluded that the bacterium was not hampered *in vivo* by intestinal mucus to establish close
447 contact with the epithelial lining of the chicken gut. Moreover, invasion *in vivo* of the
448 epithelial cells by *C. jejuni* has been observed microscopically (Welkos, 1984).
449 Shortly after internalization, it was observed that *C. jejuni* was released again from the cells in
450 the medium, after which it could re-invade the cell layer and there appeared to be a strong
451 difference between strains in their ability to do so. It is unlikely that release of the bacterium
452 from the invaded cells is the result of cell lysis, since invasion was not accompanied by
453 necrosis or apoptosis during a 24 hour infection period and since no significant loss of cells
454 was observed during the evasion assay compared with the non infected control.
455 Our results suggest that replication inside epithelial cells is not likely to be important *in vivo*
456 for persistent cecal colonization and that invasion alone can not be responsible for *C. jejuni'*s
457 persistence in chickens. Rather, a dynamic process of adherence, invasion, escape from the
458 cell layer, fast replication in mucus and re-invasion of the cell layer might explain why the
459 bacterium is not rapidly expelled from the intestine. Interestingly, while KC 40 was the most
460 invasive strain, it could not be recovered after the reinvasion assay. One explanation might be
461 that the final addition of gentamicin for 1 hour was enough to kill all evading KC 40 strains
462 due to their fast evasion rate, while the slower strains R-27456 and R-27473 remained
463 protected intracellularly.
464 *C. jejuni* interaction with its avian host seems to be commensal in character. Indeed, in our
465 cecal loop no gross inflammatory response, increase of heterophil influx, necrosis or tissue
466 damage could be observed after inoculation with *C. jejuni*, in contrast with loops inoculated
467 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,

493 and subsequent internalization *in vitro*, the colonization in chickens is devoid of any
494 inflammatory response of the chicken intestinal wall and it cannot be ruled out that *C. jejuni*
495 is capable of manipulating the chicken immune response in order to avoid destructive
496 inflammation of the gut, similarly to the immune modulation by some commensal bacteria.
497

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506

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598 **Tables**

599

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
C. jejuni 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*
C. jejuni 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
Salmonella Enteritidis	76SA88t	5.2 ± 0.1	5.1 ± 0.0	4.6 ± 0.2*
Escherichia coli	DH5α	-	-	-

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Table 2: Mean (log_{10} cfu/g ± 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 ± s.e.m.	No. of positive samples	Mean ± s.e.m.	No. of positive samples
KC40	1.4 ± 0.2	12/20	0.8 ± 0.2	7/20
R-27456	0.1 ± 0.1	1/20	0.2 ± 0.1	2/20
R-27473	0.5 ± 0.1	4/17	0.2 ± 0.1	2/17

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606 **Figure captions**

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

611

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

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618

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

623

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

628

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

631 PBS injected cecal loop (20 x) showing healthy cecal tissue. (B) Cross section of a *C. jejuni*
632 infected loop (20 x) showing healthy, normal cecal tissue. (C) Low power view (5 x) of
633 *Salmonella* Enteritidis infected loop, showing necrotic lesion affecting the deeper layer of the
634 mucosa, which is replaced by dense infiltrate of mononuclear cells lymphocytes and
635 heterophils (arrow). A purulent caseous plug can be observed in the lumen containing necrotic
636 cells, heterophils, blood and protein debris. Viable (a) and necrotic tissue (b) separated by an
637 intensely colored band consisting of heterophils and monocytes (c). (D) High power view of
638 *Salmonella* infected cecal mucosa (100 x) showing heterophil infiltration (arrowheads),
639 haemorrhages and apoptotic/necrotic enterocytes (arrow).
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641

1 **Tables**

2

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

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	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
Salmonella Enteritidis	76SA88t	5.2 ± 0.1	5.1 ± 0.0	4.6 ± 0.2*
Escherichia coli	DH5α	-	-	-

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R-27456	0.1 ± 0.1	1/20	0.2 ± 0.1	2/20
R-27473	0.5 ± 0.1	4/17	0.2 ± 0.1	2/17

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Figure 1

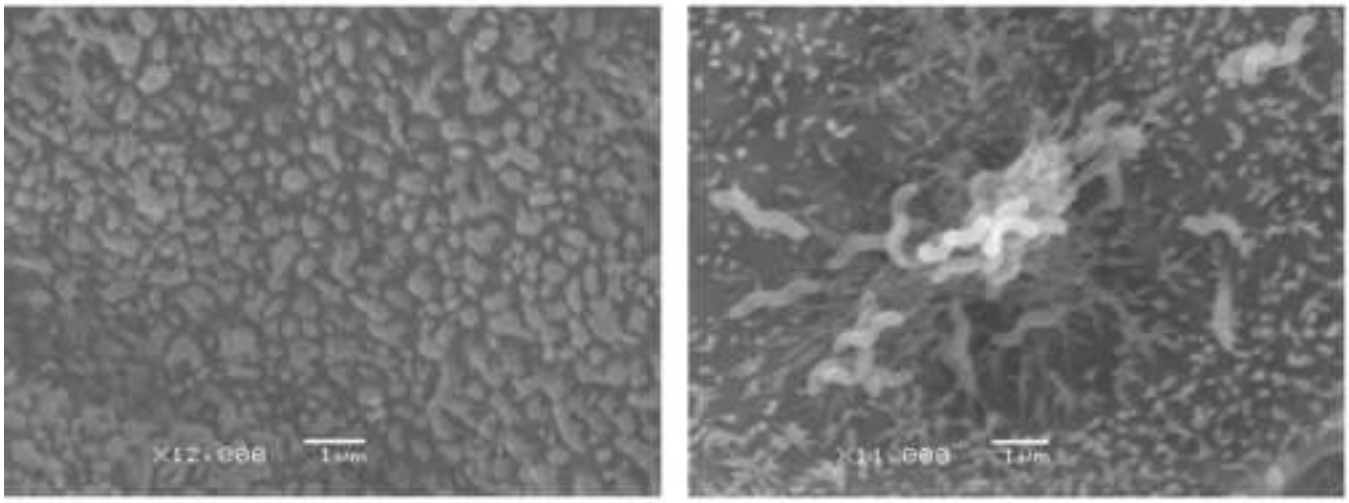

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Figure 2

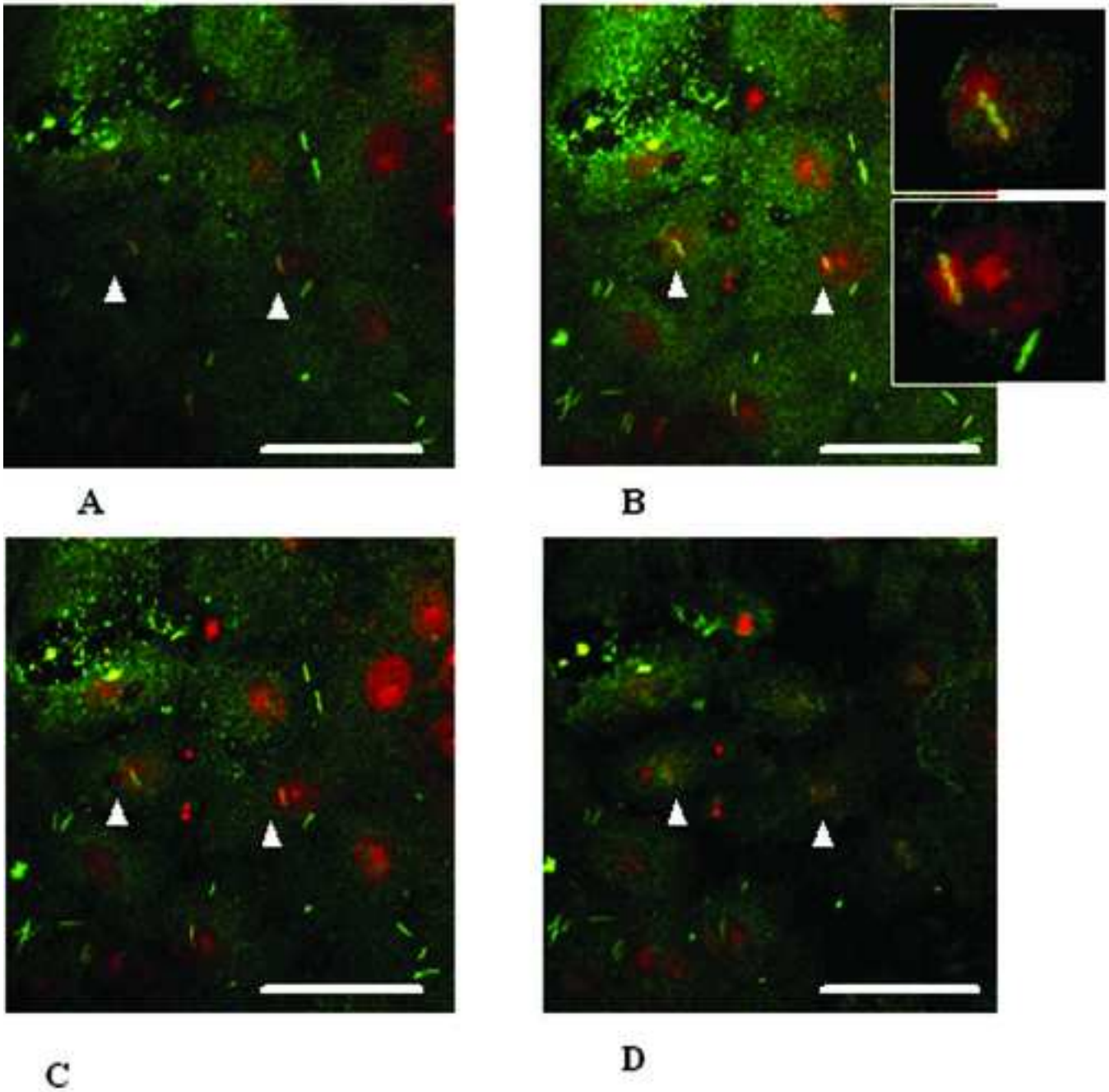

Figure 3

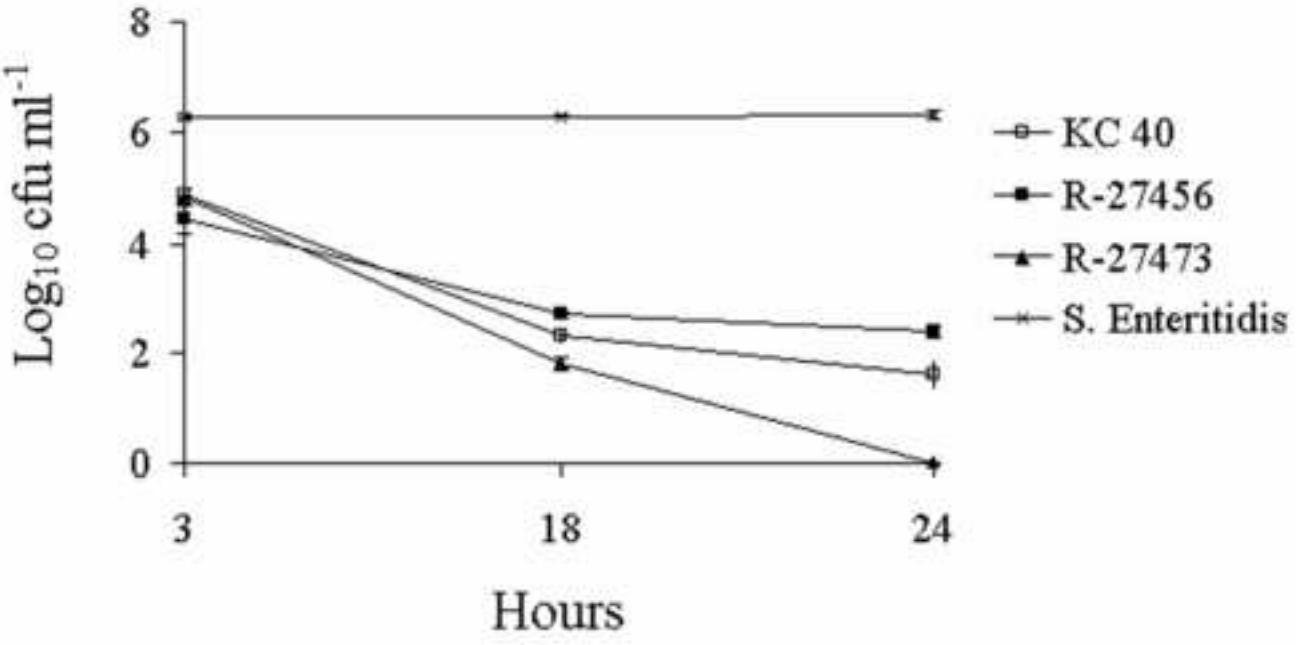

Figure 4

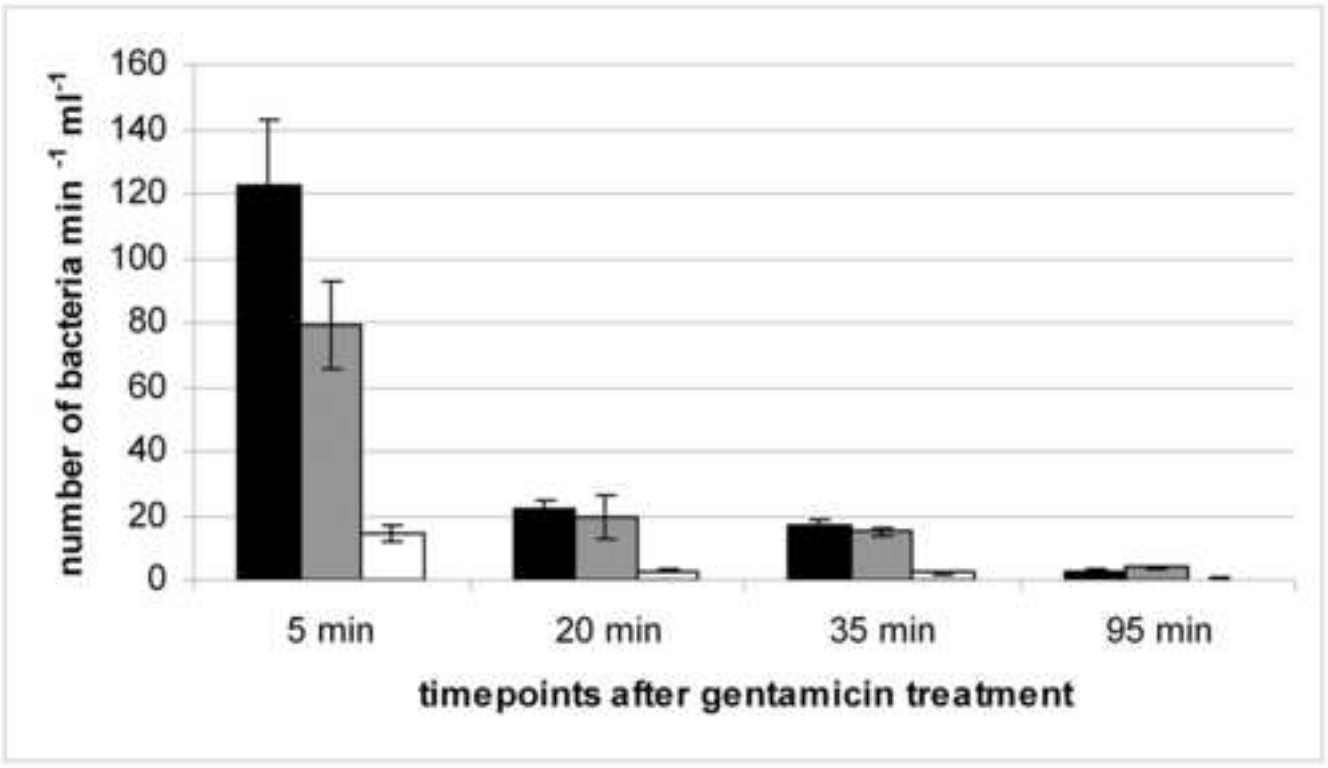

Figure 5