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## Colonization strategy of results in persistent infection of the chicken gut

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► **To cite this version:**

Kim van Deun, Frank Pasmans, Richard Ducatelle, Bram Flahou, Kris Vissenberg, et al.. Colonization strategy of results in persistent infection of the chicken gut. *Veterinary Microbiology*, 2008, 130 (3-4), pp.285. 10.1016/j.vetmic.2007.11.027 . hal-00532365

**HAL Id: hal-00532365**

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Submitted on 4 Nov 2010

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## Accepted Manuscript

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

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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

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

3

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

5

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20

## 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

46 (Wingstrand *et al.*, 2006). Although severe complications may arise from infection with *C.*  
47 *jejuni* such as the Guillain-Barré syndrome (Hughes and Cornblath, 2005), the disease in  
48 humans usually presents as a self-limiting enteritis. *C. jejuni* may belong to the normal avian  
49 microbiota and despite a huge colonization number of up to  $10^8$  colony forming units (cfu)  
50 per gram intestinal content, chickens generally remain asymptomatic (Dhillon *et al.*, 2006). In  
51 contrast with the hypothesis that *C. jejuni* is a mere commensal bacterium in chickens, some  
52 studies report the ability of *C. jejuni* to invade the chicken intestinal mucosa (Knudsen *et al.*,  
53 2006) and cause systemic infection (Sanyal *et al.*, 1984).

54 *C. jejuni* usually appears in broiler flocks at an age of two to three weeks (Gregory *et al.*,  
55 1997), which coincides with a drop in maternal antibody titers (Sahin *et al.*, 2001). It has been  
56 reported that as few as 2 to 35 cfu (Stern *et al.*, 1988; Knudsen *et al.*, 2006) are sufficient for  
57 cecal colonization, while others mention a higher minimal inoculation dose, up to  $5 \times 10^4$  cfu  
58 for 14 day old chicks (Ringoir *et al.*, 2007). The ability to colonize the chicken gut varies  
59 between strains (Young *et al.*, 1999), but once one chicken is infected, the bacterium spreads  
60 rapidly through the whole flock, resulting in infection of almost 100% of the chickens  
61 (Lindblom *et al.*, 1986). Several genes, including the *dnaJ*, *pldA* and *cadF* genes, have been  
62 associated with colonization of the intestinal tract (Zirpin *et al.*, 2001). It has been reported  
63 that intact motile flagella are necessary for efficient colonization (Nachamkin *et al.*, 1993).  
64 Wassenaar *et al.*, however, concluded that it is the presence of flagellin A rather than motility  
65 that contributes to colonization (1993). *Campylobacter* invasion antigen (Cia) proteins also  
66 play a role in chicken gut colonization (Biswas *et al.*, 2007). These proteins are secreted in the  
67 presence of chicken serum and mucus. It was the purpose of this study to examine the  
68 colonization strategy of *C. jejuni* in chickens. Therefore the interaction of the bacterium with  
69 chicken primary cecal epithelial cells was studied *in vitro*, while inflammation was examined

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

72

## 73 **2. Materials and Methods**

74

### 75 *2.1. Experimental animals*

76

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

87

### 88 *2.2. Bacterial strains and growth conditions*

89

90 *Campylobacter jejuni* strains KC 40, KC 51, KC 69.1 and KC 96.1 from poultry origin  
91 (kindly provided by Dr. Marc Heyndrickx, ILVO, Melle, Belgium) and R-27450, R-27456, R-  
92 27461 and R-27473 (kindly provided by Prof. Dr. Peter Vandamme, Ghent University, Ghent,  
93 Belgium) isolated from human patients with gastroenteritis, were used in the *in vitro* invasion  
94 assay. Based on experimental results described further in this paper, strains KC 40, R-27456

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

104

### 105 2.3. Cell lines

106

107 The human colon carcinoma cell line T84 was grown in 44% D-MEM, 44% F-12 (Gibco,  
108 Merelbeke, Belgium), 10% fetal calf serum (FCS) (Integro B.V., aa Dieren, Netherlands), 1%  
109 L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) at 37°C in 5% CO<sub>2</sub>  
110 atmosphere.

111

### 112 2.4. Isolation of chicken primary cecal epithelial cells from crypts

113

114 Ceca from commercial brown laying hens at the age of 12 to 20 weeks were used to isolate  
115 primary epithelial cells from crypts according to a modified protocol of Booth *et al.*, (1995).

116 Ceca were washed with HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup> to remove fecal content, diced and  
117 digested at 37°C in digestion medium (99% DMEM, 1% FCS, 25 µg/ml gentamicin (Gibco),  
118 100 U/ml penicillin, 100 µg/ml streptomycin, 375 U/ml collagenase (Sigma) and 1 U/ml  
119 dispase (Roche, Vilvoorde, Belgium) until crypts were found floating in the medium The

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

126

### 127 *2.5. Isolation of embryonal chicken fibroblasts*

128

129 Ten day old chicken embryos were euthanized and head, legs and wings were removed. The  
130 abdomen was diced and washed with PBS, 100 U/ml penicillin and 100 µg/ml streptomycin.  
131 The diced fragments were transferred in an Erlenmeyer with 50 ml PBS, 10% trypsin, 100  
132 U/ml penicillin and 100 µg/ml streptomycin and gently stirred. Fibroblasts were harvested by  
133 adding 5 ml FCS and subsequent centrifugation of the tissue fragments at 220 x g for 10 min  
134 at room temperature. The supernatant was discarded and the cells were suspended in a  
135 phosphate buffered solution containing 137.0 mM NaCl, 2.5 mM KCl, 8.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5  
136 mM KH<sub>2</sub>PO<sub>4</sub>, 1.0 mM MgCl<sub>2</sub>, 1.0 mM CaCl<sub>2</sub> and 20% FCS. The cell suspension was  
137 centrifuged at 220 x g for 10 min at room temperature and the supernatant was discarded. The  
138 cells were suspended in fibroblast growth medium (90% MEM, 10% FCS, 100 U/ml  
139 penicillin, 100 µg/ml streptomycin, 1% potassium, 1% L-glutamine) and seeded in a 25 cm<sup>2</sup>  
140 culture flask. When grown to full density, they were subcultured in 75 cm<sup>2</sup> culture flasks  
141 before storage at -80°C.

142

143

144



145 2.6. Adhesion of *C. jejuni* to chicken primary cecal epithelial cells

146

147 Adherence of *C. jejuni* to chicken primary cecal epithelial cells was studied using Scanning  
148 Electron Microscopy (SEM). Primary cecal epithelial crypt cells were plated on HCl treated  
149 glass coverslips in 24 well plates at a density of 2500 crypts per well. After 40 hours, cells  
150 were washed and inoculated with *C. jejuni* strain KC 40 at a multiplicity of infection (m.o.i.)  
151 of 200. At different time points, cells were gently washed once and fixed overnight in 5%  
152 paraformaldehyde, 437.0 mM NaCl, 187.0 mM HEPES, 12.5 mM CaCl<sub>2</sub>.H<sub>2</sub>O, 5.8%  
153 glutaraldehyde, pH 7.2. Samples were post fixed with 1% osmiumtetroxide for 2 hours at  
154 room temperature. After dehydration through a graded series of alcohol and acetone, samples  
155 were critical point dried (Balzers, Liechtenstein) and platinum sputter-coated with a JFC-  
156 1300 Auto fine coater (Japanese Electronic Optical Laboratories, Japan). Analysis was  
157 performed on a JSM-5600LV (Japanese Electronic Optical Laboratories, Japan) scanning  
158 electron microscope.

159

160 2.7. Invasion and intracellular survival of *C. jejuni* in chicken primary cecal epithelial cells,  
161 chicken embryonic fibroblast cells and T84 cells

162

163 To examine the contribution of invasion as a possible mechanism for persistent colonization,  
164 chicken primary cecal epithelial crypt cells and chicken embryonic fibroblast cells were  
165 inoculated with *Campylobacter*, *Salmonella* or *E. coli* DH5 $\alpha$ . The two latter served  
166 respectively as a positive and negative control for invasion. Chicken embryonic fibroblast  
167 cells were seeded in 96-well plates at a density of 10<sup>5</sup> cells per well. In all invasion assays, an  
168 m.o.i. of 200 was used. Plates were centrifuged at 500 x g for 10 min at 37°C. After 3 hours  
169 incubation at 37°C in a 5% CO<sub>2</sub> atmosphere, cells were washed with HBSS with Ca<sup>2+</sup> and

170  $Mg^{2+}$  (Gibco) to remove non-invaded bacteria. Extracellular bacteria were killed by adding  
171 100  $\mu$ g/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  $\mu$ g/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  $\mu$ M cytochalasin D (Sigma) as inhibitor for actin filament polymerization and  
193 20  $\mu$ M nocodazole (Sigma) for the inhibition of microtubule formation. *Salmonella* was used  
194 as a control, since its invasion is microfilament dependent but microtubule independent

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

199

200 Intracellular survival was assessed using chicken primary cecal epithelial crypt cells and T84  
201 cells. Invasion with *C. jejuni* strains KC 40, R-27456 and R-27473 was carried out as  
202 described in the invasion assay, but after the killing of extracellular bacteria, cells were  
203 incubated at 37°C in a 5% CO<sub>2</sub> atmosphere in cell medium containing 50 µg/ml gentamicin,  
204 to avoid extracellular replication of bacteria. The incubation time was set at 4, 18 and 24  
205 hours for the primary cecal epithelial crypt cells and 3, 18 and 24 hours for T84 cells.  
206 *Salmonella* served as a positive control for intracellular survival assay only in T84 cells,  
207 because preliminary test revealed that *Salmonella* caused massive damage to primary cecal  
208 epithelial crypt cells after 5 hours incubation. After incubation, intracellular bacteria were  
209 quantified as earlier.

210

#### 211 2.8. Evasion of *C. jejuni* from primary cecal epithelial cells

212

213 Escape from the cell layer after invasion was examined using primary chicken cecal epithelial  
214 crypt cells. An invasion assay with *C. jejuni* strains KC 40, R-27456 and R-27473 was carried  
215 out as described above. After killing of extracellular bacteria with gentamicin, cells were  
216 incubated for another 19 hours at 37°C in a 5% CO<sub>2</sub> atmosphere in 200 µl of cell medium  
217 without antibiotics. At regular time intervals (5 min, 20 min, 35 min, 95 min, 3 hours, 6 hours,  
218 10 hours, 12 hours and 19 hours), the medium was replaced with fresh medium. The collected

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

221

### 222 2.9. Reinvasion of *C. jejuni* from primary cecal epithelial cells

223

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

233

### 234 2.10. Apoptosis / necrosis assay

235

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

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

250

### 251 *2.11. Growth of C. jejuni in chicken intestinal mucus and intestinal contents*

252

253 To assess the survival and replication capacity of *C. jejuni* in chicken intestinal mucus, the  
254 small intestine of commercial 20 weeks old brown laying hens was collected and gently  
255 rinsed with PBS to remove fecal material. The mucus was scraped from the mucosa with a  
256 scalpel, diluted 1/3 with HEPES (N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid, 25  
257 mM; pH 7.4) and vortexed. The solution was centrifuged three times at 1000 x g for 10 min at  
258 4°C. The mucus was filter sterilized by passage through a 0.45 µm pore size filter (IWAKI,  
259 International Medical, Brussels, Belgium) and stored at -80°C. Five ml phosphate buffered  
260 saline (PBS) supplemented with 5 mg/ml mucus protein was inoculated with  $1 \times 10^7$  cfu/ml of  
261 strain KC 40, R-27456 or R-27473. The controls consisted of PBS, BHI and BHI  
262 supplemented with 5 mg/ml bovine serum albumin (BSA). At different time points, bacterial  
263 counts were made by titration on mCCDA.

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

268

269 2.12. Colonization of day of hatch chickens with *C. jejuni* strains

270

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

283

284 2.13. Cecal loop model

285

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

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

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

305

#### 306 2.14. Statistical analysis

307

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

312

### 313 3. Results

314

#### 315 3.1. *C. jejuni* is able to adhere to and invade in chicken primary cecal epithelial cells

316

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

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

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

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

339 *3.2. Invasion in chicken primary cecal epithelial cells is predominantly microtubule*  
340 *dependent*

341

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



344 8 *C. jejuni* strains was inhibited by 20  $\mu$ M nocodazole. Remarkably, treatment with 2  $\mu$ M  
345 cytochalasin D increased invasion for two strains. Poultry strain KC 40 was inhibited by both  
346 nocodazole and cytochalasin, while invasion of strains KC 51 and R-27473 was not  
347 influenced by any of the inhibitors. Results are summarized in table 1.

348

349 *3.3. C. jejuni is not able to survive intracellularly in chicken primary cecal epithelial cells*

350

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

358

359 *3.4. C. jejuni can evade and reinvade chicken primary cecal epithelial cells without inducing*  
360 *cytotoxicity*

361

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

369 to the number of intracellular bacteria as determined in the invasion assay, 65% of strain R-  
370 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  
371 recovered, whereas KC 40 was not.

372

373 *3.5. C. jejuni is able to survive and multiply in chicken intestinal mucus but not in intestinal*  
374 *contents*

375

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

386

387 *3.6. Invasion of chicken primary epithelial cecal cells by C. jejuni is not correlated with cecal*  
388 *colonization and inflammation in chickens, but does correlate with spleen colonization*

389

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

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

478 The *C. jejuni* strain dependent differences in invasiveness in the chicken cecal epithelial cells  
479 were not correlated with *in vivo* colonization of the ceca of experimentally infected chickens,  
480 but rather with systemic colonization. Indeed, strain KC 40, a highly invasive strain *in vitro*,  
481 was recovered from more spleen and liver samples and in higher numbers than the medium  
482 and low invasive strains. This finding is in contrast with the observations of Hänel *et al.*  
483 (2004), who found a correlation between colonization phenotype and Caco-2 invasion, and  
484 this could be due to variation between the cells used.

485

## 486 **Conclusion**

487 In conclusion, our data suggest a colonization mechanism whereby *C. jejuni* is able to avoid  
488 being expelled from the chicken gut by temporal invasion and evasion of the crypt epithelial  
489 cells and persists through rapid multiplication in the mucus. This close interaction with the  
490 epithelial lining of the cecum is accompanied by translocation of the bacterium to spleen and  
491 liver, whereby invasion of spleen and liver correlates with the *in vitro* invasion capacity in  
492 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

#### 498 **Acknowledgements**

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

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

506

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598 **Tables**

599

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

		Mean (log <sub>10</sub> cfu/ml ± s.e.m.) of intracellular bacteria.								
		* denotes values significantly different ( <i>P</i> < 0.05) from the values in the absence of the inhibitor								
Origin	Strain	Without inhibitor			With 20 μM nocodazole			With 2 μM cytochalasin		
		<i>C. jejuni</i> poultry	KC 40	3.4	±	0.1	2.5	±	0.2*	2.9
	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	-			-			-		

600

601

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

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  $\mu\text{m}$ , (B) 0.9  $\mu\text{m}$ , (C) 1.2  $\mu\text{m}$  and (D) 2.4  $\mu\text{m}$   
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  $\mu\text{m}$ .

617

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  $\pm$   
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  $\pm$  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).

640

641

1 **Tables**

2

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

Mean ( $\log_{10}$  cfu/ml  $\pm$  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 $\mu$ M nocodazole			With 2 $\mu$ M cytochalasin		
		Mean	SE	CI	Mean	SE	CI	Mean	SE	CI
<i>C. jejuni</i> poultry	KC 40	3.4	$\pm$ 0.1		2.5	$\pm$ 0.2*		2.9	$\pm$ 0.2*	
	KC 51	2.2	$\pm$ 0.2		1.7	$\pm$ 0.4		2.4	$\pm$ 0.4	
	KC 69.1	2.6	$\pm$ 0.2		0.9	$\pm$ 0.3*		2.3	$\pm$ 0.3	
	KC 96.1	2.1	$\pm$ 0.2		1.6	$\pm$ 0.3		2.9	$\pm$ 0.1*	
<i>C. jejuni</i> human	R-27450	2.4	$\pm$ 0.1		1.2	$\pm$ 0.3*		2.9	$\pm$ 0.2*	
	R-27456	2.7	$\pm$ 0.2		1.6	$\pm$ 0.3*		2.8	$\pm$ 0.3	
	R-27461	1.9	$\pm$ 0.2		0.8	$\pm$ 0.2*		1.3	$\pm$ 0.3	
	R-27473	2.1	$\pm$ 0.2		2.1	$\pm$ 0.3		2.4	$\pm$ 0.2	
<i>Salmonella</i> Enteritidis	76SA88t	5.2	$\pm$ 0.1		5.1	$\pm$ 0.0		4.6	$\pm$ 0.2*	
<i>Escherichia coli</i>	DH5 $\alpha$	-			-			-		

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

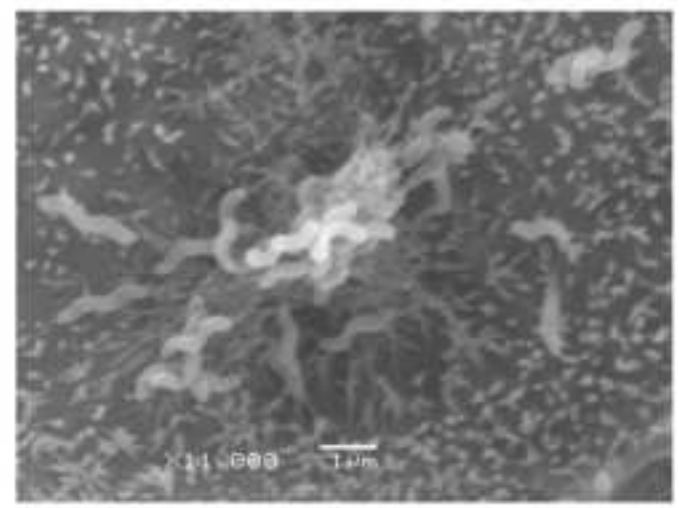
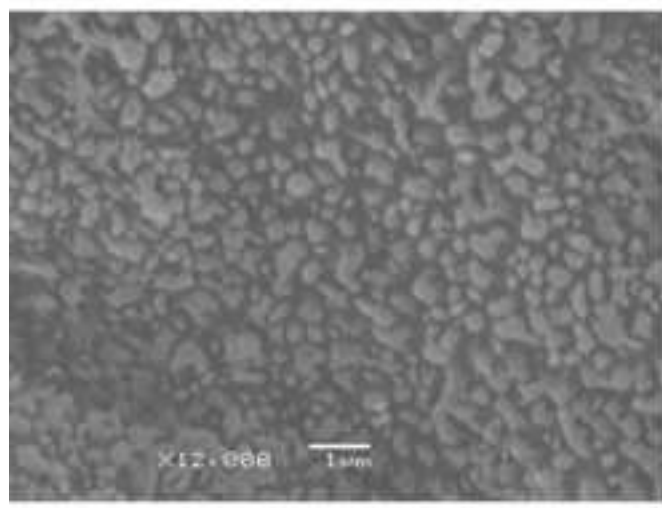
6

7

8

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Figure 1

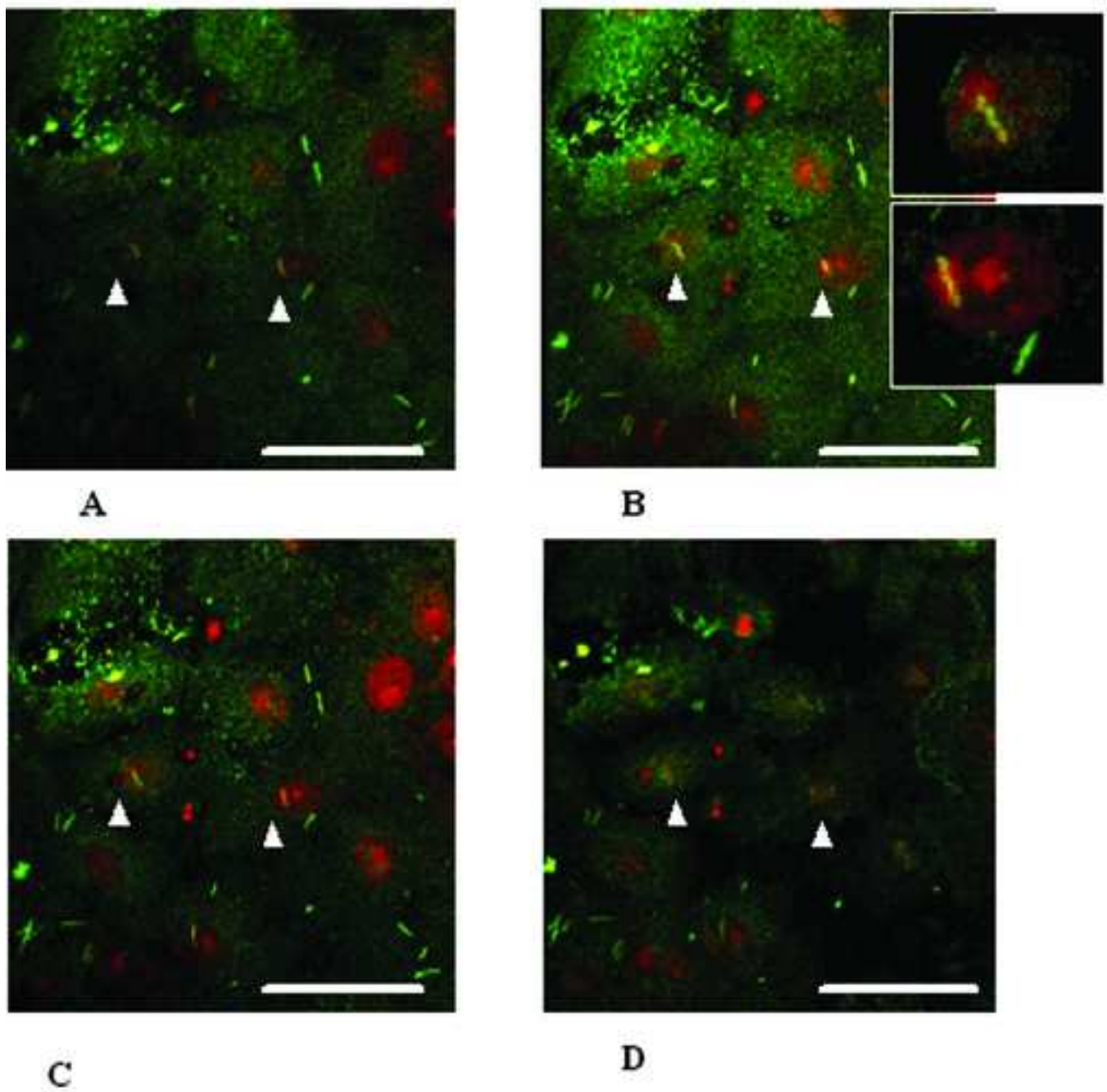


**A**

**B**



Figure 2



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Figure 3

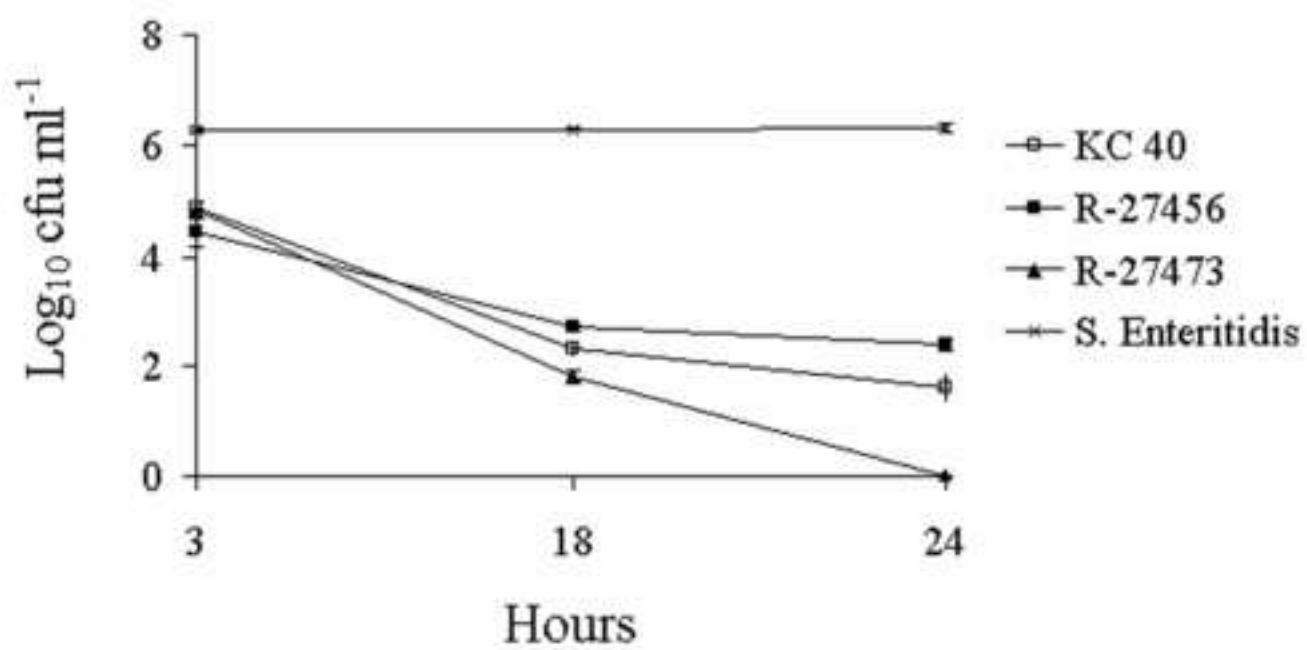
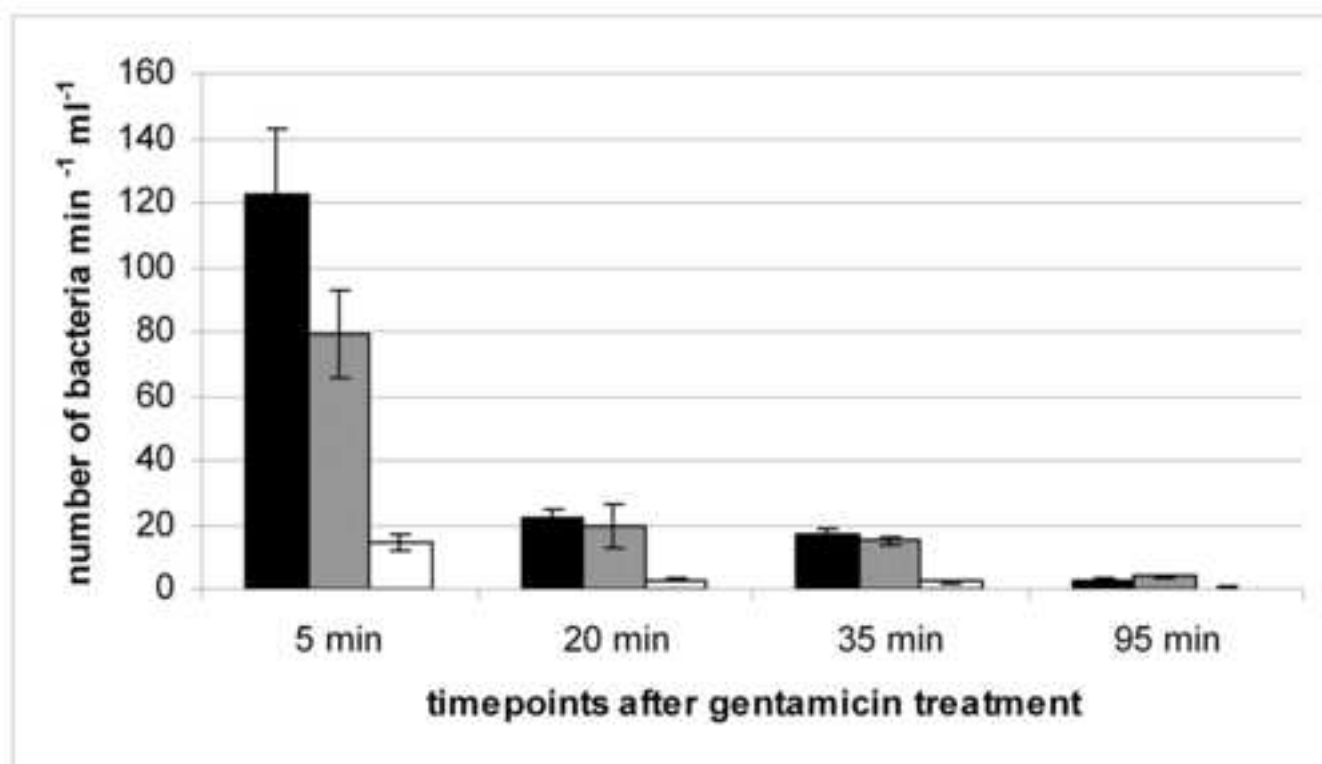


Figure 4



Manuscript

Figure 5

