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1 *Galleria mellonella* as an infection model for *Campylobacter jejuni* virulence

2

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Running title: *C. jejuni* insect model

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Models of infection

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49 **Summary**

50 Larvae of *Galleria mellonella* (Greater wax moth) have been shown to be
51 susceptible to *Campylobacter jejuni* infection and our study characterises this
52 infection model. Following infection with *C. jejuni* human isolates, bacteria
53 were visible in the hemocoel and gut of challenged larvae, and there was
54 extensive damage to the gut. Bacteria were found in the extracellular and cell
55 associated fraction in the hemocoel, and we show that *C. jejuni* can survive in
56 insect cells. Finally, we have used the model to screen a further sixty-seven
57 *C. jejuni* isolates belonging to different MLST types. Isolates belonging to
58 ST257 were the most virulent, whereas those belonging to ST21 were the
59 least virulent in the *Galleria* model.

60

61 **Introduction**

62 *Campylobacter jejuni* is recognised as the leading cause of bacterial
63 gastroenteritis across the developed world; the World Health Organisation
64 estimates that 1% of the population of Western Europe is infected with
65 campylobacters each year. It is thought that for each reported case, a further
66 nine go unreported (Humphrey *et al.*, 2007), thus, based on the reported
67 figures for 2009 from the Health Protection Agency, this would mean there
68 were in excess of 500,000 cases in England and Wales alone. Furthermore,
69 beyond the initial diarrhoeal disease, *C. jejuni* may also cause post-infection
70 complications including irritable bowel syndrome, meningitis and Guillain-
71 Barré Syndrome, plus its variant Miller-Fisher Syndrome (Janssen *et al.*,
72 2008; van Doorn *et al.*, 2008).

73

74 Despite having been first identified as a causative agent of diarrhoea in 1977
75 (Skirrow, 1977), *C. jejuni* pathobiology remains poorly understood, with its
76 core virulence determinants remaining elusive. A major contributing factor in
77 the lack of determination of these factors has been the absence of a suitable
78 infection model for *C. jejuni*. Previous models have included a ferret
79 diarrhoeal model (Fox *et al.*, 1987), a chick colonisation model (Wassenaar *et*
80 *al.*, 1993) and a colostrum deprived piglet model (Babakhani *et al.*, 1993).
81 However, in common with other mammalian and avian models, their
82 widespread use has been limited by factors such as cost, ease of use,
83 reproducibility and ethics (Newell, 2001).

84

85 We recently reported that larvae of the lepidopteran insect *Galleria mellonella*
86 (Greater wax moth) are susceptible to infection by *C. jejuni* and can be used
87 to screen for virulence genes (Champion *et al.*, 2010). In this study we aimed
88 to characterise *G. mellonella* as a *C. jejuni* infection model and to screen a
89 panel of multi locus sequence typed (MLST) *C. jejuni* field isolates for
90 virulence in *G. mellonella*. Different MLST groups were chosen to cover types
91 detected in the main food producing animals, the environment and clinical
92 disease.

93

94 **Methods**

95 **Strains and cultures**

96 All bacterial strains and mutants used in this study are shown in Table 1. *C.*
97 *jejuni* strain 11168H is a hypermotile variant of the sequenced strain
98 NCTC11168 that readily colonises chickens (Jones *et al.*, 2004; Karlyshev *et*

99 *al.*, 2002). *C. jejuni* strains were cultured on either blood agar Skirrows
100 actidione (BASA) plates or Columbia agar plates (CBA) supplemented with
101 5% (v/v) horse blood in anaerobic jars in an atmosphere of 6% O₂/10% CO₂
102 conditions (CampyPak, Oxoid) for 48 hours at 37°C.

103

104 For infections, bacteria were subcultured into 6ml of Mueller-Hinton (MH)
105 broth (Oxoid) and grown under microaerobic conditions for 24 to 48 hours at
106 37°C, 150rpm. The bacteria were then adjusted to OD_{590nm} 1.0 in phosphate-
107 buffered saline (PBS, 0.1M, pH 7.2) for infections, equivalent to 1 x 10⁸ cfu/ml.
108 Infections at lower doses were adjusted accordingly.

109

110 ***G. mellonella* virulence assays**

111 *G. mellonella* larvae were purchased from Live Foods UK and maintained on
112 wood chips at 15°C. The infection of larvae was carried out as previously
113 described (Champion *et al.*, 2010) using a micro-injection technique whereby
114 10µl of *C. jejuni* was injected into the hemocoel via the right fore leg, using a
115 Hamilton syringe. Larvae were then incubated at 37°C and survival and
116 macroscopic appearance recorded at 24 hours post-infection. PBS injected
117 and uninfected controls were used. For each, experimental groups of ten *G.*
118 *mellonella* larvae were infected.

119

120 **The association of *C. jejuni* with hemocytes**

121 A group of three *G. mellonella* larvae was infected as above with 10⁶ cfu of *C.*
122 *jejuni* 11168-H and incubated at 37°C for 24 hours. The larvae were chilled
123 on ice for 20 minutes before aseptic removal of the bottom 2mm of the body.

124 The hemocoel was drained from each larva into sterile microcentrifuge tubes
125 and centrifuged at 200 x g for 5 minutes. The supernatant, which was the
126 hemolymph, was transferred to a separate sterile microcentrifuge tube. The
127 pelleted hemocytes were resuspended in 100µl of sterile distilled water, and
128 pipetted up and down ten times to lyse the cells. Serial dilutions of both
129 hemolymph and hemocytes were plated out on CBA to enumerate bacteria.

130

131 **Histopathology**

132 *C. jejuni*-infected and uninfected larvae (5 per group) were fixed by immersion
133 in 10% (v/v) neutral buffered formalin for 3-7 days. For light microscopy,
134 larvae were blocked by a longitudinal section dividing the animal into two
135 pieces and smears were air-dried and stained with Gram-Twort. The larvae
136 (20%) were blocked into eleven transversal sections serially from the cranial
137 to the caudal extremities of the larvae. Sections were embedded in paraffin
138 wax and routinely stained with Haematoxylin and Eosin (H&E) for microscopic
139 examination.

140

141 **Investigation of *C. jejuni* morphology following infection of *G. mellonella***

142 A GFP-tagged *C. jejuni* strain, pREM5 11168H GFP (donated by Andrey
143 Karlyshev) was cultured under microaerobic conditions on MH agar. It was
144 then subcultured into MH broth as before and incubated for 24 hours at 37°C
145 under microaerobic conditions. An inoculum was prepared at OD_{590nm} as
146 previously described above. Five *G. mellonella* larvae were infected with 10
147 µl of the prepared inoculum, and a further five were inoculated with 10 µl of
148 PBS. The larvae were incubated at 37°C for 3 hours before being chilled on

149 ice for 5 minutes. They were then swabbed with 70% ethanol prior to the
150 aseptic removal of the bottom 2mm of the body as previously described. One
151 of each larval set was drained separately; the other 4 of each set had their
152 hemocoel combined. This combined hemocoel was centrifuged at 500 x *g* for
153 5 minutes to pellet the hemocytes, and 10 µl of the supernatant (hemolymph)
154 was dropped onto a slide. A further 10 µl from the non-centrifuged hemocoel
155 and 10 µl from the overnight *C. jejuni* culture were also dropped onto separate
156 slides. Slides were examined using a Zeiss LSM 510 META confocal
157 microscope.

158

159 **Cell culture**

160 J774A.1, a murine monocyte macrophage-like cell line, was obtained from the
161 American Type Culture Collection (ATCC), (Reference TIB-67), and was
162 cultured in Dulbecco's modified eagle medium (DMEM) supplemented with
163 10% fetal bovine serum at 37°C. SF9, a lepidopteran cell line, was donated
164 by Richard French-Constant, and cultured in Grace's Insect Medium (GIM)
165 supplemented with 10% fetal bovine serum at 27°C. The cells were seeded at
166 2×10^5 cells in 6-well tissue culture plates and then incubated at the
167 appropriate temperature for 24 hours under 5% CO₂ prior to infection with *C.*
168 *jejuni*.

169

170 **Bacterial infection of cultured cells**

171 *C. jejuni* 11168-H was cultured on a CBA plate and harvested from an
172 overnight culture by rolling a moistened swab over the plate; cells were re-
173 suspended in PBS. OD_{590nm} was measured and the inoculum was prepared

174 at a Multiplicity of Infection (MOI) of 10 in L-15 medium before being added to
175 both macrophages and insect cells (3 replicates). The macrophages were
176 incubated at 37°C for 1 hour; the insect cells were incubated at 27°C for 1
177 hour. Following incubation, the inoculum in each well was replaced with L-15
178 medium containing 50 µg ml⁻¹ gentamicin, and the plates then incubated at
179 the appropriate temperature for a further 1 hour. The medium was removed
180 and the cells incubated in L-15 containing 10 µg ml⁻¹ gentamicin for
181 approximately 16 hours.

182

183 The cells were then washed x3 with PBS, and 1ml cold sterile water was
184 added to each well. The cells were mechanically lysed to release intracellular
185 bacteria, and colony forming units (cfu) were determined after plating out
186 serial dilutions on CBA plates and incubating microaerobically at 37°C.

187

188 **Results**

189 ***C. jejuni* induces histopathological changes in *G. mellonella***

190 In order to better understand the fate of *C. jejuni* inoculated into *G. mellonella*,
191 larvae challenged with 10⁶ cfu of some well-characterised human isolates
192 were fixed in neutral buffered formalin at 24 hours post-infection and
193 sectioned for histopathology. Figure 1 shows H and E stained sections of
194 uninfected and infected larvae. Bacteria were observed in the hemocoel and
195 sections of gut from infected larvae, but were absent in sections from
196 uninfected controls. There was evidence of damage to the midgut, with
197 apoptotic cells and loss of integrity to the gut wall in the infected larvae. This
198 damage was not visible in the control sections. Other tissues (fat body,

199 muscle, nervous tissue) appeared undamaged in infected larvae and
200 uninfected controls. Pigmented nodules were also present in infected larvae,
201 and bacteria were associated with these nodules. These nodules were not
202 visible in the uninfected control larvae.

203

204 The observed bacteria were coccoid rather than having the characteristic
205 spiral form associated with *C. jejuni*. In order to investigate whether these
206 coccoid bacteria were actually *C. jejuni*, *G. mellonella* larvae were infected
207 with GFP-tagged *C. jejuni*; the hemocoel was collected and centrifuged at low
208 speed to sediment hemocytes, which are often auto-fluorescent.
209 Comparisons with *C. jejuni* from an overnight culture showed that these
210 bacteria had the expected morphology, but bacteria in hemolymph were of a
211 coccoid nature (Figure 2). Similar observations of hemolymph from control
212 larvae inoculated with PBS showed no fluorescence at all.

213

214 To investigate the site of replication, hemocoel was collected and centrifuged
215 at low speed to sediment hemocytes. The number of bacteria found in the re-
216 suspended cell pellet (4.1×10^6 cfus; s.e.m 2.98×10^6) was broadly similar to
217 the number found in the hemolymph (7.7×10^6 cfus; s.e.m 2.3×10^6).

218

219 A macro scoring system was used to examine whether there was a correlation
220 between the colour of the larvae and the presence of bacteria in the gut or
221 body cavity (Table 2; Figure 3). There was a significant association between
222 macro colour and the presence of bacteria in the body cavity ($p < 0.001$,
223 Kruskal-Wallis non-parametric test). No associations were made between the

224 location of the bacteria and temperature at which it had been grown, or
225 location and the strain of *C. jejuni* used to inoculate the larvae.

226

227 **An insect cell line and mammalian macrophages are comparable in their**
228 **response to challenge with *C. jejuni***

229 Insect (SF9) and mammalian (J774A.1) cells were infected at an MOI of 10
230 with *C. jejuni* 11168-H, and monitored at 4 hours and 24 hours post-infection.

231 In J774A.1 macrophages, bacterial numbers declined 100-fold by 4 hours
232 post-infection (Figure 4). However, the bacterial numbers then remained
233 approximately constant in the macrophages at 24 hours post-infection. There
234 was a broadly similar pattern of survival in the SF9 cell line. Bacterial
235 numbers decreased 1000-fold during the first 4 hours of the infection, but
236 there was an approximate 10-fold increase in bacterial numbers between 4
237 hours and 24 hours (Figure 4). This increase was statistically analysed using
238 a Student's t-test and found to be significant ($p < 0.05$).

239

240 **Differences in virulence were observed in *G. mellonella* between *C.***
241 ***jejuni* Multi-Locus Sequence Typing (MLST) complexes**

242 To investigate whether there was an association between MLST type and
243 virulence, larval survival was recorded following challenge with sixty-seven *C.*
244 *jejuni* strains belonging to different MLST types (Figure 5). It was observed
245 that there was variation within MLST groups as well as between them. There
246 was a significant difference ($p = 0.0002$) between the ability of ST21 and
247 ST257 strains to cause disease. Overall, strains belonging to ST21 showed

248 the least virulence in the model, whilst strains belonging to ST257 were the
249 most virulent.

250

251 **Discussion**

252 We have previously demonstrated that *G. mellonella* larvae can be used to
253 screen for virulence of *Campylobacter* genes (Champion *et al.*, 2010). In this
254 study we have characterised the *G. mellonella* model and demonstrated that it
255 can be used as an infection model to provide data about pathology and
256 intracellular survival.

257

258 Similarities between invertebrate and mammalian humoral and cellular innate
259 immune responses are exploited when using insects as virulence models. For
260 example, *G. mellonella* possesses a cuticle that acts in the same physical
261 barrier capacity as mammalian skin (Kemp & Massey, 2007). Once the
262 cuticle has been breached, *G. mellonella* induces a humoral response,
263 producing soluble factors such as antimicrobial peptides (Mullett *et al.*, 1993).
264 In parallel with a humoral response, *G. mellonella* induces a cellular response
265 to invading micro-organisms. Insect hemocytes phagocytose bacteria in a
266 manner similar to that of mammalian neutrophils and produce a respiratory
267 burst (Bergin *et al.*, 2005). Thus, the response of *G. mellonella* to infection
268 with *C. jejuni* is likely to have similarities to the response of humans.

269

270 In the initial experiment, larvae were infected with human *C. jejuni* isolates
271 11168-H, 11168-O, 81116, 81176 and 01/51. These were selected as they
272 are well-characterised in a number of other animal models; invasion and toxin

273 data are also available for them. It would not have been possible to fix *G.*
274 *mellonella* for all the strains used later on, as this would have been time
275 consuming and expensive with no guarantee of any further data.

276

277 Histopathology of infected larvae demonstrated that bacteria are found in the
278 hemocoel and in the gut and that extensive tissue damage occurs in the latter.
279 This pathology may be caused by hemocytes in the gut tissue, which have
280 ingested bacteria circulating in the hemocoel and then produced responses
281 such as the release of free radicals and peroxide, causing the visible tissue
282 damage. The presence of pigmented nodules, which are aggregations of
283 hemocytes around foreign bodies, indicates a vigorous immune response to
284 infection (Lackie, 1980). The observed colour change in infected larvae
285 correlating with the presence of bacteria in the body cavity is a product of
286 melanogenesis; this process is thought to protect endogenous tissues within
287 the cavity from systemic damage resulting from pathogen killing (Nappi &
288 Christensen, 2005).

289

290 The bacteria observed in the larval sections were not identified, but were only
291 present in *Campylobacter* infected larvae. The bacterial cells were coccoid
292 rather than spiral; however, *C. jejuni* that have become intracellular convert
293 rapidly from the spiral form to the coccoid form (Kiehlbauch *et al.*, 1985).
294 There is some debate about how this change affects the bacteria (Moore,
295 2001). Adaptation to the coccoid form is generally seen as a response to
296 stress, such as starvation or oxidative stress (Harvey & Leach, 1998). Some
297 studies, such as Moran & Upton (1986), have reported that the coccoid form is

298 thus degenerative. However, it has also been reported that coccoid *C. jejuni*
299 become viable but non-culturable, with the potential to still act as an infectious
300 agent (He & Chen, 2010). To ascertain whether these coccoid cells were
301 likely to be *C. jejuni*, a comparison was made between GFP-tagged *C. jejuni*
302 grown in broth overnight versus the same bacteria inoculated into *G.*
303 *mellonella* and incubated for 3 hours. Under confocal microscopy, the
304 fluorescing bacteria from broth were seen to be elongated, reflecting the
305 normal spiral morphology of *C. jejuni*; the bacteria from the larval hemolymph
306 were short and round like the coccoid bacteria seen in the larval sections.
307 Hemolymph from PBS control larvae contained no fluorescing bacteria. This
308 observation suggests that the coccoid bacteria are indeed *C. jejuni*.

309

310 Within the hemocoel, cell-associated and free bacteria were found. *C. jejuni*
311 is primarily an extracellular pathogen; however, intracellular survival
312 has been hypothesised to play an important role in its pathogenesis
313 (Kiehlbauch *et al.*, 1985; Hickey *et al.*, 2005; Young *et al.*, 2007).
314 Reproducible *in vitro* infection models that mimic pathogenesis *in vivo* have
315 been used to study *C. jejuni* intracellular survival in epithelial cells (De Melo *et*
316 *al.*, 1989; Watson & Galan, 2008). However, reports of *C. jejuni*
317 intramacrophage survival *in vitro* are conflicting. Some groups indicate that *C.*
318 *jejuni* is killed by macrophages (Watson & Galan, 2008); others suggest that
319 the bacteria survive within the macrophage (Day *et al.*, 2000; Hickey *et al.*,
320 2005). In this study, bacterial infection of different cell lines was undertaken to
321 establish whether there was a difference in response between mammalian
322 macrophages and an insect cell line. It was uncertain as to whether *C. jejuni*

323 would survive intracellularly in the SF9 insect cell line under tissue culture
324 conditions. However, although the levels of *C. jejuni* recovered from the
325 insect cell line were approximately ten times lower than those recovered from
326 murine macrophages at 4 hours post-infection, it is clear from the data
327 presented here that the bacteria did invade the cells and survive within them.

328

329 The bacterial numbers recovered from the macrophages remained consistent
330 between 4 hours and 24 hours; there was survival within the macrophages.
331 This is consistent with previous studies (Kiehlbauch *et al.*, 1985; Hickey *et al.*,
332 2005). However, there was a significant increase in recovered *C. jejuni* from
333 the insect cell line at 24 hours compared to 4 hours. Thus, it is possible that
334 the bacteria not only survived within the cells, but also replicated.

335

336 These observations are consistent with the hypothesis that *C. jejuni* enters
337 insect hemocytes during *in vivo* model infections of *G. mellonella*. This
338 intracellular persistence may allow the bacteria to avoid, or at least reduce the
339 impact of, host antimicrobial defences. Nevertheless, the fact that at least
340 some bacteria provoked the formation of melanised nodules is not surprising
341 as it has previously been shown that nodule formation is associated with
342 phagocytosis (Dean *et al.*, 2004).

343

344 *C. jejuni* strains can be classified by MLST complexes. A number of studies
345 have sought to establish whether there is a link between MLST type and the
346 development of post-infectious complications (Dingle *et al.*, 2001; Nielsen *et*
347 *al.*, 2009; Islam *et al.*, 2009). It was noted that the ST-22 complex is

348 overrepresented in isolates from patients who have contracted Guillain-Barré
349 Syndrome; no Guillain-Barré related isolates have been shown to carry ST-45,
350 despite it being a common sequence type (Dingle *et al.*, 2001; Nielsen *et al.*,
351 2009). No sequence types have been found to be exclusive for clinical
352 outcomes (Islam *et al.*, 2009). This supports the findings of Manning *et al.*
353 (2003), who studied a large number of *C. jejuni* isolates and found that in
354 terms of MLST types, the populations of veterinary and human isolates
355 overlapped; it was suggested that most veterinary sources should be
356 considered reservoirs of pathogenic campylobacters. However, these studies
357 did not assess whether bacteria from different MLST types exhibited different
358 levels of virulence. Recent studies have suggested that there may be
359 associations between *C. jejuni* MLST type and virulence factors (Habib *et al.*,
360 2009; de Haan *et al.*, 2010). We observed that when bacteria selected as
361 representatives of major MLST groups were put through the *G. mellonella*
362 model, MLST type 257 strains were significantly more virulent than the MLST
363 type 21 set. MLST type 257 is mainly associated with poultry and clinical
364 isolates. MLST type 21 is common in all food producing animals; the strains
365 used here are also all of clonal complex (CC) 21, which is one of the four most
366 common CCs in human disease. The reduced virulence of these isolates in
367 the model may thus appear anomalous, but Habib *et al.* (2010) suggest that
368 the abundant prevalence of *C. jejuni* of CC-21 may be a result of its increased
369 tolerance of stresses encountered during the human food chain. A less
370 virulent but more stress tolerant strain would thus be encountered more
371 frequently than a more virulent strain that did not tolerate such stresses to the
372 same extent. The convenience of the *G. mellonella* model allows for high

373 throughput screening to assay for the differences in virulence. Such a model
374 could provide preliminary data when considering food security issues.

375

376 This study has sought to further characterise *G. mellonella* as a model for *C.*
377 *jejuni* infection, and suggests that, since the bacteria convert to a coccoid form
378 once within the insect, it may be used to provide opportunities for further study
379 of this morphological change. The model may also prove useful in
380 investigating the *in vivo* intracellular survival of *C. jejuni* within macrophages,
381 an area of some dispute. In particular, the model allows screening for natural
382 variations in the virulence of *C. jejuni* field isolates, which would prove
383 invaluable for tracking particularly virulent strains in the food chain.

384

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388

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498 Tables

499

500	Strain	Origin	MLST group
501			
502	11168-H	Human	ST43/CC21
503	11168-O	Human	ST43/CC21
504	pREM5 11168H	Human	ST43/CC21
505	81116	Human	ST267/CC283
506	81176	Human	ST913/CC42

507	01/51	Human	ST626/CC21
508	01/43	Human	ST257/CC257
509	93/372	Pet	ST21/CC21
510	94/229	Poultry	ST45/CC45
511	99/97	Human	ST45/CC45
512	99/118	Cow	ST21/CC21
513	99/188	Human	ST21/CC21
514	99/189	Human	ST45/CC45
515	99/194	Cow	ST45/CC45
516	99/197	Human	ST45/CC45
517	99/201	Cow	ST48/CC48
518	99/202	Cow	ST45/CC45
519	99/212	Human	ST45/CC45
520	99/216	Human	ST45/CC45
521	99/242	Poultry	ST45/CC45
522	A1/CF/12	Poultry	ST257/CC257
523	A6/T2/15	Poultry	ST257/CC257
524	A8/35/15A	Poultry	ST257/CC257
525	C1/C/2	Poultry	ST257/CC257
526	C120/2	Poultry	ST257/CC257
527	C132/1	Poultry	ST19/CC21
528	C3/T/25	Poultry	ST257/CC257
529	C5/T/2/8	Poultry	ST257/CC257
530	C85-4-99-5	Cow	ST262/CC21
531	C500-1-99-2	Cow	ST48/CC48
532	C559-3-99-2	Cow	ST262/CC21
533	D2/27/3	Poultry	ST48/CC48
534	D2/T/8	Poultry	ST48/CC48
535	D2/T/95	Poultry	ST48/CC48
536	D5-20-9A	Poultry	ST262/CC21
537	EX1182	Environmental	ST262/CC21
538	EX1286	Poultry	ST262/CC21
539	MB1	Poultry	ST48/CC48
540	MB2	Poultry	ST48/CC48
541	MB3	Poultry	ST48/CC48
542	MB4	Poultry	ST19/CC21
543	MB5	Poultry	ST19/CC21
544	MB6	Poultry	ST19/CC21
545	MB7	Poultry	ST262/CC21
546	MB8	Poultry	ST48/CC48
547	MB9	Poultry	ST257/CC257
548	MB10	Poultry	ST19/CC21
549	MB12	Poultry	ST21/CC21
550	MB13	Poultry	ST21/CC21
551	MB14	Poultry	ST21/CC21
552	MB15	Poultry	ST45/CC45
553	MB16	Poultry	ST48/CC48
554	MB17	Poultry	ST262/CC21
555	MB18	Poultry	ST21/CC21
556	Ps308	Pig	ST51/CC403
557	Ps549.1	Pig	ST403/CC403
558	Ps623	Pig	ST552/CC403
559	Ps762	Pig	ST270/CC403
560	Ps830	Pig	ST403/CC403
561	Ps838	Pig	ST403/CC403
562	Ps843	Pig	ST403/CC403
563	Ps849	Pig	ST403/CC403
564	Ps852	Pig	ST270/CC403
565	Ps857	Pig	ST270/CC403
566	S39-2-99-3	Sheep	ST21/CC21
567	S87-4-99-3	Sheep	ST262/CC21
568	S120-4-99-4	Sheep	ST45/CC45
569	S216-5-99-1	Sheep	ST257/CC257
570	S372-5-99-4	Sheep	ST21/CC21
571	S379-8-99-1	Sheep	ST262/CC21
572	S435-3-99	Sheep	ST262/CC21
573	S499-1-99-5	Sheep	ST19/CC21
574	S585-3-99	Sheep	ST19/CC21

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576 Table 1: A table describing the bacterial strains used in this study

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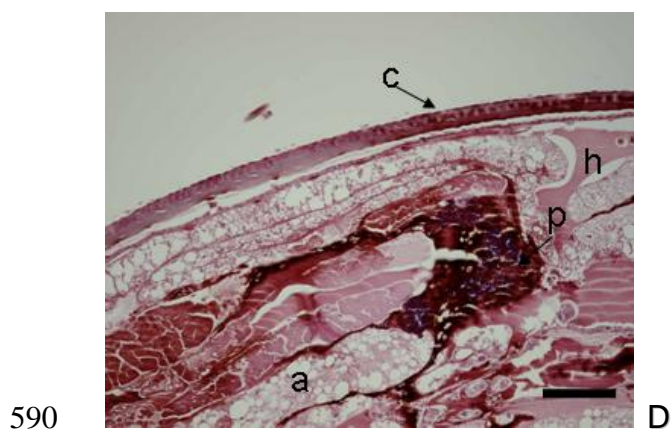
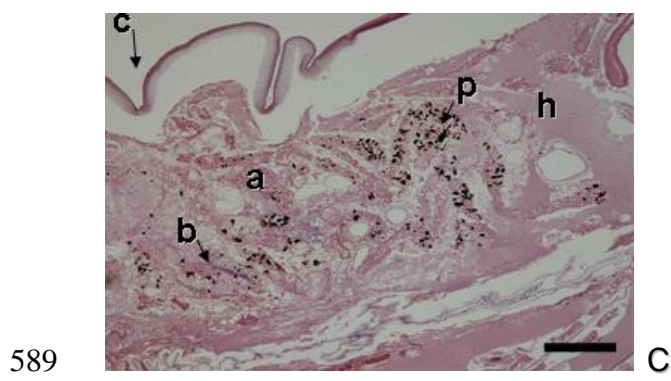
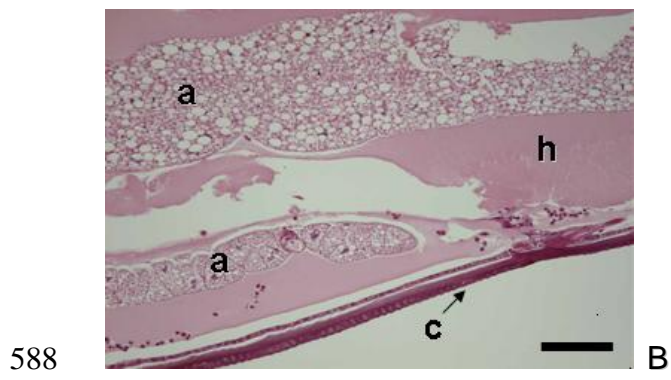
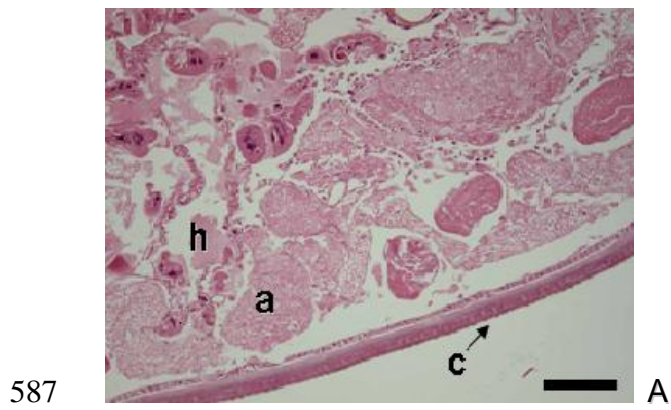
<i>C. jejuni</i> strain	Incubation temperature	Animal number	Macro colour	Bacteria in gut	Bacteria in cavity	Mean <i>Galleria</i> survival
01/51	37°C	1	0	3	0	80%
		2	2	3	3	
		3	3	3	3	
		4	3	3	3	
		5	3	2	2	
	42°C	1	2	3	3	82%
		2	2	3	2	
		3	2	2	1	
		4	2	3	3	
		5	2	3	3	
11168-O	37°C	1	1	2	3	27%
		2	1	3	3	
		3	2	3	3	
		4	2	3	3	
		5	3	3	3	
	42°C	1	1	3	2	80%
		2	2	2	2	
		3	2	1	2	
		4	2	3	3	
		5	2	3	3	
11168-H	37°C	1	1	2	3	0%
		2	1	2	1	
		3	2	3	3	
		4	2	1	0	
		5	3	3	2	
	42°C	1	0	2	0	ND
		2	1	3	1	
		3	1	2	1	
		4	2	3	1	
		5	3	3	3	
81116	37°C	1	1	2	0	90%
		2	1	3	0	
		3	3	3	3	
		4	3	3	3	
		5	3	3	3	
	42°C	1	1	2	2	65%
		2	1	2	0	
		3	2	3	2	
		4	2	2	0	
		5	3	1	3	
81176	37°C	1	1	2	0	90%
		2	1	2	0	
		3	2	2	0	
		4	2	2	1	
		5	3	2	3	
	42°C	1	2	2	2	75%
		2	2	3	3	
		3	2	3	3	
		4	3	2	3	
		5	3	3	3	
PBS		1	0	1	0	100%
		2	0	2	0	
		3	0	2	0	
		4	0	2	0	
		5	1	1	0	
Uninfected		1	0	1	0	100%
		2	0	1	0	
		3	0	1	0	
		4	0	2	0	
		5*	1	3	3	

580

581 Table 2: Macro scores for different *C. jejuni* strains in terms of colour, presence of
582 bacteria in the larval gut and presence of bacteria in the larval body cavity. *G.*
583 *mellonella* were incubated at different temperatures. * autolysis. A score of 3 for
584 macro colour refers to fatality. ND = no data.

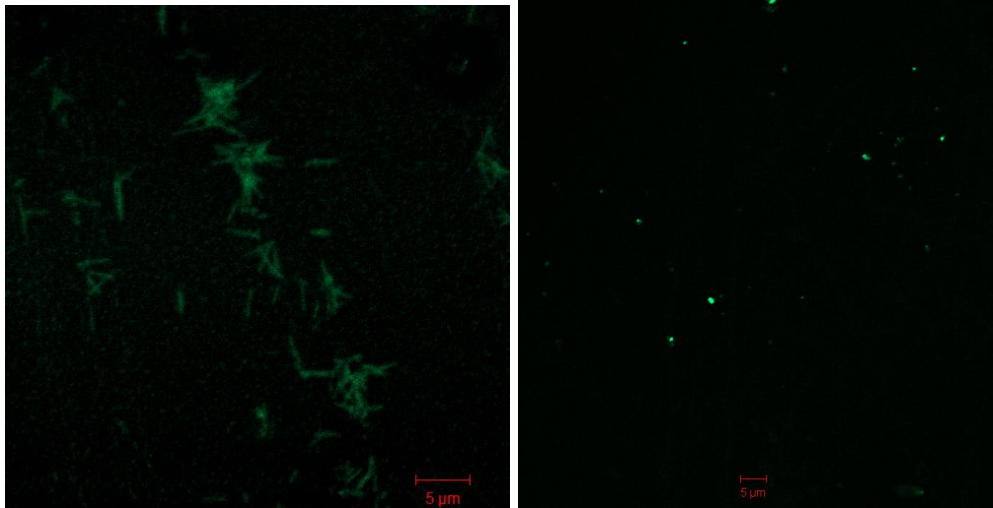
585

586 **Figures**



591

592 Figure 1: H&E stained sections of *Galleria mellonella*. (A) Non-infected larva (Bar:
593 100µm). (B) Control [0.1M PBS inoculation] larva. (Bar: 100µm) (C) Larva infected
594 with *C. jejuni* 81116 and incubated at 37°C (Bar: 250µm). (D) Larva infected with *C.*
595 *jejuni* 81116 and incubated at 42°C (Bar: 100µm). Structures annotated as follows: a
– adipose bodies, b – bacterial colonies, c – cuticle, h – hemolymph, m – muscle, p –
pigmented structures.



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597

598 Figure 2: confocal microscope images of GFP-tagged *C. jejuni* pREM5 11168H.
599 Left: *C. jejuni* from an overnight broth culture. Right: *C. jejuni* in the
hemolymph of infected *G. mellonella* after 3 hours' incubation.

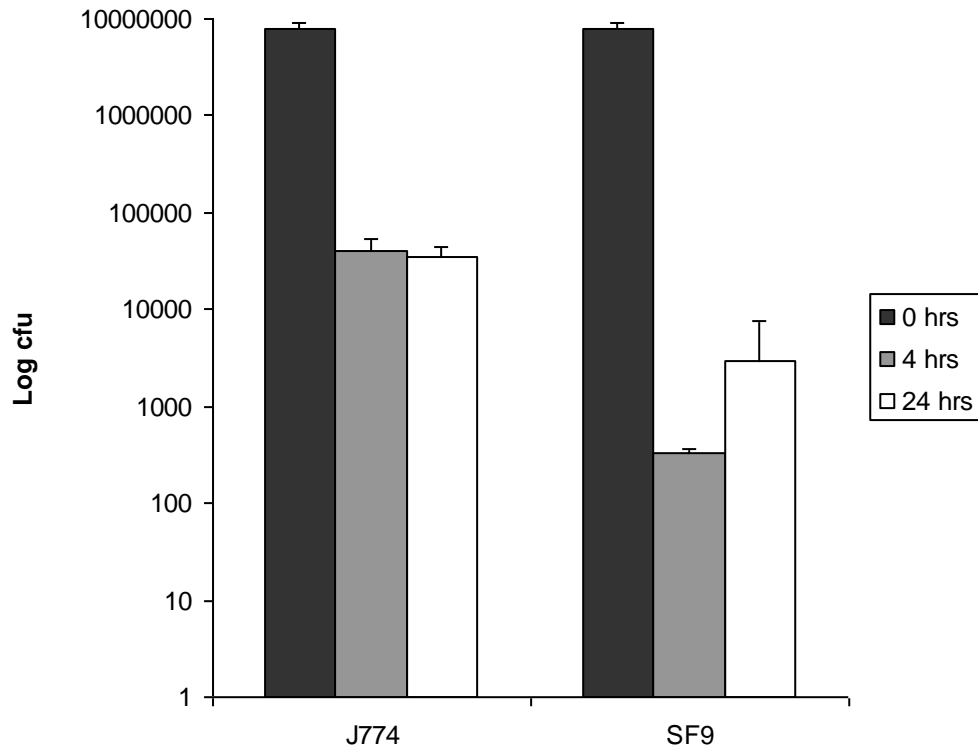
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602 Figure 3: Macroscopic evaluation of wax moth larvae following infection with
C. jejuni: white, score 0; orange, score 1; black, score 3



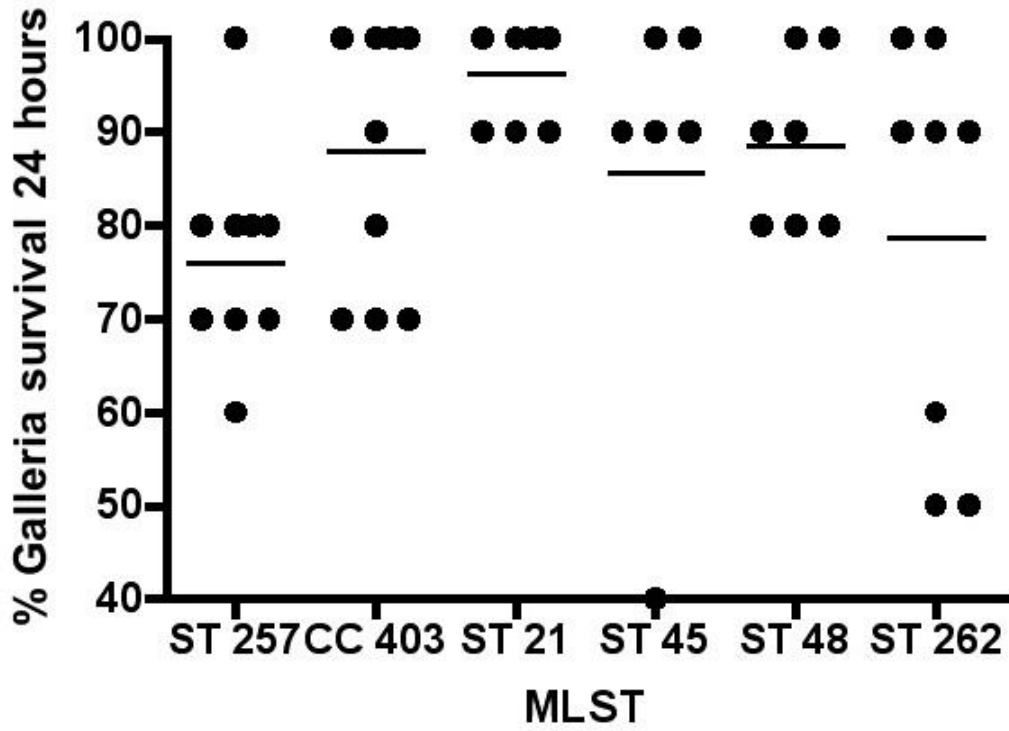
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Figure 4: A chart to show the average numbers of *C. jejuni* 11168-H recovered from J774.1A murine macrophages and SF9 insect cell line at 4 hrs and 24 hrs post-infection (n = 3). 0 hrs represents the initial inoculum.

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Figure 5: A graph of data from MLST infections of *G. mellonella*. Each spot represents the average percentage larval survival (n = 10). Horizontal lines represent the mean for each MLST type.