Pancreatic Amylase is an Environmental Signal for Regulation of Biofilm Formation and Host Interaction in *Campylobacter jejuni*

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Abstract

Campylobacter jejuni is a commensal bacterium in the intestines of animals and birds and a major cause of foodborne gastroenteritis in humans worldwide. Here we show that exposure to pancreatic amylase leads to secretion of an α-dextran by *C. jejuni* and that a secreted protease, Cj0511, is required. Exposure of *C. jejuni* to pancreatic amylase promotes biofilm formation *in vitro*, increases interaction with human epithelial cell lines, increases virulence in the *Galleria mellonella* infection model and promotes colonisation of the chicken ileum. We also show that exposure to pancreatic amylase protects *C. jejuni* from stress conditions *in vitro* suggesting that the induced α-dextran may be important during transmission between hosts. This is the first evidence that pancreatic amylase functions as an inter-kingdom signal in an enteric micro-organism.

Introduction

Campylobacter infection is a major cause of foodborne gastroenteritis in humans worldwide (1). Disease ranges from mild, non-inflammatory, self-limiting diarrhea to prolonged, inflammatory diarrhea and occasional serious complications such as Guillain-Barré syndrome and reactive arthritis (1). Ninety percent of human disease is attributed to *C. jejuni*, while *Campylobacter coli* accounts for the remainder (2).

In contrast to human infection, *C. jejuni* establishes a largely asymptomatic but persistent infection in animals and birds, and the major risk of human infection is from handling and consumption of poultry meat (3, 4, 5). Campylobacters primarily inhabit the lower gastrointestinal (GI) tract of poultry and contamination of the flesh

of the bird occurs at slaughter. *Campylobacter* spp. are present in the luminal crypts of the chicken gut as densely-packed communities surrounded by mucus (6), an arrangement suggestive of a biofilm, a structured community of bacteria enclosed in a self-produced exopolymeric matrix (EPM) composed primarily of polysaccharides (7). Biofilms have also been described on experimentally-infected human ileum *ex vivo* suggesting that this mode of growth is also important during human infection (8, 9).

Despite several studies of *C. jejuni* biofilms (10-15), the EPM has not been characterised. Polysaccharide is a major component of most bacterial EPMs and provides the structural framework (7, 16, 17). *C. jejuni* produces several surface-associated carbohydrate structures including lipooligosaccharide (LOS), capsular polysaccharide (CPS) and both *N*- and *O*-linked glycoproteins (18), but there is no evidence that these glycans contribute to biofilm formation (11, 19).

Enteric bacteria use environmental signals present in host environments to regulate the expression of components required for interaction with the host and signals include bile salts, mucus, low oxygen, bicarbonate and neuroendocrine stress hormones (20-22). *C. jejuni* shows increased interaction with intestinal epithelial cells (IECs) in response to low oxygen, bile salts and noradrenaline (23-25). In addition, bile salts have recently been reported to enhance *C. jejuni* biofilm formation by causing release of extracellular DNA (26).

Here we show that *C. jejuni* responds to the presence of pancreatic amylase by secreting an α-dextran that is a component of the biofilm EPM. This is the first definitive characterisation of the polysaccharide component of *C. jejuni* biofilms. Importantly, pre-exposure to pancreatic amylase results in significant changes in the

interactions of *C. jejuni* with the host in both *in-vitro* and *in-vivo* infection models. Pre-exposure to pancreatic amylase also promotes stress tolerance *in vitro* suggesting that the α-dextran may be important during transmission. This is the first evidence that pancreatic amylase is used by an enteric micro-organism as a signal to regulate interaction with the host.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains are listed in Table 1. *C. jejuni* was stored at -70ºC in Brucella broth (BB; Oxoid) containing 15% glycerol and was grown on Mueller Hinton agar (MHA; Oxoid), Brucella agar (BA; Oxoid), Columbia blood agar (CBA; Oxoid) containing 7% defibrinated horse blood (E & O Laboratories) in a microaerobic atmosphere generated using Campypaks (Oxoid) in gas jars at 37°C. Charcoal (0.4%) was added to BA to improve contrast for photography. For broth cultures, BB or Eagle's Minimal Essential Medium-α (MEM-α; Sigma-Aldrich, M0894) were used and incubated with shaking (50 rpm) at 37°C in 5% CO_2. Kanamycin (Km; 40 µg/ml) and chloramphenicol (Cm; 20 µg/ml) were used for selection.

Sources of pancreatic amylase. Hog pancreatic α-amylase (HPA) was purchased from Sigma Aldrich. Polyhistidine-tagged human pancreatic amylase expressed and purified from human cells was obtained from Sino Biologicals. For chicken pancreas extract, 20 organs were homogenized in 0.05 M Tris, 0.9% NaCl, 0.05 M $CaCl_2$, pH

8.0 (27). After centrifugation (1,000 x *g*, 20 min), amylase activity in the supernatant was quantified by enzymatic assay (28).

EPM purification and characterisation. Growth (48 h) removed from MHA with or without 100 nM HPA was suspended in phosphate buffered saline (PBS) and the extracellular material was recovered using modifications of a method previously used to isolate exo-polysaccharide from *Vibrio parahaemolyticus* (29). Briefly, the bacterial cell suspensions in PBS were washed on a rotary platform at 200 rpm for 1.5 h at 30ºC, vortexed for 15 min and washed again at 200 rpm for 1.5 h at 20ºC. The supernatant was recovered (1,800 x *g*, 15 min) and precipitated overnight with 4 volumes of cold acetone at 4°C. The precipitate was recovered (450 x *g*, 5 min), washed in distilled water (dH_2O), dried (Speedvac SPD1010, Thermo Scientific), dissolved in dH_2O and stored at -70°C. Total carbohydrate was measured by phenol-sulphuric acid assay (30) with glucose as standard. To determine the statistical significance, a one-way ANOVA was used to compare the amount of secreted carbohydrate in response to the seven different doses of pancreatic amylase. Within the one-way ANOVA we constructed two contrasts, the first compared the joint effect of the three lowest doses with the joint effect of the four highest doses, the second compared the effect of a dose of 50 nM pancreatic amylase with 100 nM. As independent samples of a bacterial culture were exposed to the different doses of amylase in a closed system, we assumed independence between the seven experiments. To analyse the amount of carbohydrate secreted by the wild-type, *Cj0511* mutant and complemented strain, a two-way ANOVA was performed comparing the amount of secreted carbohydrate in response to the four different

doses of pancreatic amylase in the three different strains. Contrasts of the 15 nM joint with 50 nM concentrations, versus 100 nM joint with 500 nM concentrations were constructed for *C. jejuni* 11168H, *Cj0511* mutant, and complemented strains. Differences between these three contrasts were tested for statistical significance. The contrasts and comparisons were planned *a priori* and thus no multiple testing adjustments were required.

Further characterisation of the EPM carbohydrate was performed after dialysis (14 kDa MWCO) for 72 h at 4^{o}C against three changes of dH_2O per day. The EPM was freeze dried and characterized using NMR spectroscopy (31), monomer analysis (32) and linkage analysis (33) in comparison with a commercial α-dextran standard (Sigma-Aldrich).

Genetic complementation of a *cj0511* mutant. A *cj0511* mutant obtained from a collection at the London School of Hygiene and Tropical Medicine (Table 1) was used to construct a complemented strain by insertion of *cj0511* under the control of the iron-inducible *fdxA* promoter into a pseudogene (*cj0046*) in the *cj0511* mutant as previously described (34). The oligonucleotides used are listed in Table 2.

Production of recombinant Cj0511 and proteolysis assay

Codon optimized *cj0511* was synthesized by Celtek, USA and obtained in pGH. The gene was cut by HindIII (New England Biolabs, UK) and the released DNA fragment was cloned in pET-28a(+)(Novagen, UK) to generate pMAR31. Expression of *cj0511*

was achieved in *E.coli* BL21 (DE3) (New England Biolabs, UK). To localize the recombinant protein in the periplasm of *E.coli*, recombinant Cj0511 was modified to have a signal peptide from the *E.coli* DsbA protein fused to the N-terminal, while the C-terminus was fused to a 6 x-His affinity tag. Recombinant Cj0511 was purified using nickel affinity column (Qiagen) and eluted in 500 mM imidazole, 300 mM sodium phosphate and 20 mM sodium chloride buffer. The eluted fractions were combined and concentrated using an Amicon ultrafiltration column with a 100 kDa cut off (Merck Millipore, UK) then resuspended in 10 mM sodium phosphate buffer. One microgram of purified r-Cj0511 was mixed with 2 μg polyhistidine-tagged human pancreatic amylase (Sino Biologicals) or casein (Sigma Aldrich) in 20 μL of 50 mM sodium phosphate buffer (pH 7.5) and incubated at 37°C for a total of 2 h. The reactions were stopped using 6 x Laemmli buffer and the reaction mixtures were boiled for 10 min before being separated by SDS-PAGE (16% polyacrylamide) and stained with Coomassie R-250.

Biofilm assays. A 16 h culture of *C. jejuni* in BB was diluted to OD_{600} of 0.1 in BB with or without 100 nM HPA, 100 nM recombinant human pancreatic amylase or an equivalent activity of chicken pancreas extract, added to a borosilicate glass tube (VWR International) and incubated without shaking at 37°C in 5% CO_2. After 48 h the culture was decanted, the biofilms were washed with dH_2O, dried and stained for 5 min with 0.1% (w/v) crystal violet (CV; Sigma-Aldrich), washed with dH_2O and dried. Bound CV was solubilised in 80% ethanol-20% acetone and quantified by measuring OD_{600}. Statistical significance was assessed with a two-sample t-test assuming unequal variance.

Confocal laser scanning microscopy of biofilm. A 16 h culture of *C. jejuni* was diluted to OD_{600} of 0.1 in BB with or without 100 nM HPA and placed in a 6-well tissue culture plate (Sarstedt). Two glass coverslips were placed upright in each well and incubated for 48 h at 37°C in 5% CO_2. The coverslips were washed in PBS, stained with Live/Dead BacLight stain (Invitrogen) according to the manufacturer's instructions and visualised with a BioRad Radiance 2100 confocal scanning system attached to an Olympus BX51 upright microscope. Digital images were produced using Image J software (National Institutes of Health).

Adhesion and invasion of Caco-2 cells. Human colon cancer cells (Caco-2) were cultured as previously described (25) and 10^8 bacteria, grown on MHA with or without 100 nM HPA, were added at a multiplicity of infection (MOI) of 100:1 and incubated at 37°C in 5% CO_2. Interacting and invading bacteria were enumerated at 3, 6 and 24 h as previously described (25) and statistical significance was assessed using a two-sample t-test assuming unequal variance.

Interaction with T84 human colonic epithelial cells. T84 cells were grown in complete Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Invitrogen) in rat collagen pre-coated transwell dishes until the transepithelial electrical resistance (TEER) measurement (35) was >800 Ω confirming formation of tight-junctions in the monolayer. Co-culture studies were performed at a MOI of 10:1 in DMEM/F12 HAM medium containing 10% foetal bovine serum for 6 or 24 h.

184 Bacteria were grown on MHA with or without 100 nM HPA and viable counts were
185 performed. Statistical significance was assessed using a Wilcoxon matched paired
186 test. IL-8 levels were determined by ELISA (eBioscience) from the supernatant of the
187 apical surface at 24 h post-infection. Statistical significance was assessed using a
188 one-way ANOVA.

189

190 ***Galleria mellonella* infection model.** *G. mellonella* larvae were obtained
191 commercially (Cornish Crispa Co) and stored in containers with wood chips at 12°C.
192 *C. jejuni* strains were grown on MHA with or without 100 nM HPA for 48 h, harvested
193 in PBS and 10^6 cfu injected into the right foreleg of 10 *Galleria* larvae as previously
194 described (36). Statistical significance was assessed using a two-sample t-test,
195 assuming unequal variance.

196

197 **Infection of chickens.** Day-old Ross broiler chickens were obtained from a
198 commercial supplier and housed in biosecure housing. The study was performed
199 under a project licence issued by the UK Home Office under the Animals (Scientific
200 Procedures) Act 1986 (PPL 30/2599) and protocols and euthanasia techniques were
201 approved by the University of Bristol Animal Welfare Ethical Review Board. Birds
202 were fed commercial starter and grower diets (BOCM Pauls Ltd, Ipswich), had
203 access to food and water *ad libitum* and were exposed to a 12 h light/darkness cycle.
204 At 21 days old, groups of 30 birds were infected by oral gavage with 10^5 of *C. jejuni*
205 11168H or the *cj0511* mutant grown on MHA with or without 100 μM HPA for 48 h
206 and resuspended in MH broth. Fifteen birds were euthanized by cervical dislocation

at 4 and 7 days post infection and ileum and liver samples removed for culture. Ileum samples were serially diluted and plated on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid) incubated at 37^{o}C for 48 h in microaerobic conditions. Liver samples were homogenised and enriched in modified Exeter broth (37), incubated with minimal headspace at 37^{o}C for 48 h and plated on mCCDA. Differences in the number of birds colonised were assessed using a Chi-square test. The distributions of numbers of bacteria found in samples from the birds were checked for normality using a D'Agostino and Pearson omnibus normality test; as data were not shown to follow a Gaussian distribution, differences in the number of bacteria found at each site were analysed using a Kruskal-Wallis test with Dunn's multiple comparison test, using a Benjamini and Hochberg correction to adjust for multiple pairwise comparisons, as a post-hoc test.

Resistance to environmental stress. The growth from 48 h MHA plates with and without 100 nM HPA was recovered into PBS (4°C) or MH broth (20°C), washed and resuspended to an OD_{600} of 2.0. The cultures were incubated with shaking (50 rpm) at 37°C in 5% CO_2 and samples were removed at intervals for viable counting. The statistical significance of the difference in viable counts in the presence and absence of HPA was assessed using two sample t-tests, assuming unequal variance.

RESULTS

***C. jejuni* responds to the presence of pancreatic amylase.** Physiological concentrations of mammalian pancreatic α-amylase are estimated to be nanomolar (38). When hog pancreatic α-amylase (HPA) was incorporated into agar at various

concentrations, we observed the formation of large mucoid colonies of *C. jejuni* 11168H at a minimum concentration of 100 μM (Fig. 1A). It is important to note that mucoid colonies were apparent on MHA and also on BA containing 100 μM HPA; since BA does not contain starch, this indicates that the response is independent of amylase starch degrading activity. Whilst 100 μM pancreatic amylase seem high compared to the estimated *in vivo* concentrations, it is noteworthy that the number of molecules of amylase that can interact with the bacteria growing on an agar plate is likely to be low because of spatial confinement, in contrast to physiological conditions where bacteria will be bathed in litres of pancreatic juice per day. Mucoid colonies were also produced by other *C. jejuni* strains (81-176, 81116, G1, X) in response to 100 μM HPA but not by the periodontal pathogen, *Campylobacter rectus* NCTC 11489 (data not shown).

Pancreatic amylase promotes *C. jejuni* growth. As the colonies on amylase were larger in addition to being mucoid, the number of bacteria per colony was determined for *C. jejuni* 11168H from HPA-containing (100 μM) and amylase-free MHA. In three experiments, colonies contained a mean of 5.3 (+/-3.2) x 10^7 cfu in the presence of amylase, compared to 3.3 (+/-2.3) x 10^6 cfu in the absence of amylase, representing a 16-fold increase. In MEM-α, supplementation with physiological concentrations (100 nM) of HPA resulted in an increased growth rate (mean generation time of 180 (+/-0) minutes in the presence of pancreatic amylase compared to 280 (+/-35) minutes in its absence). The difference in growth rate was statistically significant ($p<0.001$) at both mid-exponential phase (10 h) and stationary phase (15 h) (Fig. 1B).

Mucoid colonies secrete increased amounts of carbohydrate. As mucoid colonies are suggestive of exo-polysaccharide production, we attempted to purify the extracellular material from colonies of strain 11168H grown in the presence of increasing concentrations of HPA by washing in PBS. Measurement of the carbohydrate content of this preparation by phenol sulphuric acid assay showed that exposure to physiological concentrations of HPA (100 nM) resulted in a 100% increase in the soluble carbohydrate secreted by *C. jejuni* 11168H compared to growth without HPA (Fig. 2A). A 11168H *kpsM* mutant, unable to export CPS (39), and 11168H *waaF* and *waaC* mutants lacking the outer and entire core oligosaccharide of LOS, respectively (40, 41), also showed increased carbohydrate secretion of comparable magnitude to the wild-type (WT) strain in the presence of HPA, indicating that the secreted carbohydrate is independent of both CPS and LOS (Fig. 2B). The amount of carbohydrate secreted by the 11168H *kpsM*, *waaC* and *waaF* mutants did not differ significantly from the wild-type strain in either the presence or absence of pancreatic amylase.

The secreted carbohydrate is an α-dextran. The soluble extracellular material recovered from *C. jejuni* 11168H and the *kpsM* mutant provided ^{1}H and ^{13}C-NMR spectra identical to an α-dextran standard (Fig. 2C) and distinct from starch (Fig. S1). A series of 2D-NMR (COSY, HSQC & HMBC) were also recorded (data not shown) to confirm the identity of the exo-polysaccharide as an α-dextran. Monomer analysis was undertaken using high performance anion exchange chromatography in combination with pulsed amperometric detection. After acid hydrolysis, the extracellular material recovered from both *C. jejuni* 11168H and the *kpsM* mutant

gave chromatographs containing a single peak which co-eluted with a glucose reference standard (Fig. S2). Monomer analysis thus confirmed that glucose was the only monosaccharide present and linkage analysis showed exclusively 1,6-glycosidic links (Fig. S3).

Cj0511 is essential for the response to pancreatic amylase but does not degrade it *in vitro*. Since HPA improves the growth of *C. jejuni*, we reasoned that it could be degraded by the bacterium. As Cj0511 is a protease (42) that is known to be secreted in outer membrane vesicles (OMVs) (43) and is abundant in a *C. jejuni* strain that colonises chickens efficiently (44), we hypothesized that it is involved in the response to pancreatic amylase. Determination of the amount of secreted carbohydrate showed that the increased secretion seen in the WT strain in response to stimulation with HPA was absent in a *cj0511* mutant (Fig. 2D). Restoration of the response to HPA in the complemented strain (Fig. 2D), shows that the defect in the *cj0511* mutant is the result of inactivation of *cj0511* and not due to a polar effect or spontaneous mutation elsewhere in the genome. To determine if Cj0511 degrades pancreatic amylase, recombinant Cj0511 (r-Cj0511) was incubated with recombinant human pancreatic amylase or with casein (Fig. 3). Partial and complete degradation of casein was apparent at 30 min and 2 h, respectively. In contrast, there was no apparent degradation of pancreatic amylase after 2 h.

Exposure to pancreatic amylase promotes biofilm formation. To determine if exposure to pancreatic amylase contributed to biofilm formation in *C. jejuni*, biofilms

formed at the air/liquid interface in glass tubes in the presence or absence of physiological concentrations of pancreatic amylase (100 nM) were measured by crystal violet staining. With the exception of the *cj0511* mutant, all the strains showed significant increases in biofilm formation in the presence of HPA (Fig. 4A). The magnitude of the increase in biofilm in response to HPA in the *waaC*, *waaF* and *kpsM* mutants was similar to the WT (2.1-2.5-fold) demonstrating that it is independent of LOS and CPS. Although we observed increased biofilm formation by the *cj0511* mutant compared to the WT in the absence of HPA ($p=0.003$), there was no significant increase in the presence of HPA in this mutant (in contrast with the complemented strain), demonstrating that a functional Cj0511 protease is required for the response (Fig. 4A). To confirm that the increased biofilm was specifically due to pancreatic amylase, the assay was repeated in the presence of 100 nM recombinant human pancreatic amylase and an increase in biofilm density of similar magnitude (2.2-fold; $p=0.001$) to that obtained with the preparation of HPA was observed (Fig. 4B). Addition of a volume of chicken pancreas extract containing equivalent amylase activity also resulted in an increase in biofilm density (2.2-fold; $p<0.001$) (Fig. 4C).

Confocal laser scanning microscopy (CLSM) was used to visualise biofilms of *C. jejuni* 11168H grown in the presence or absence of HPA. In the presence of HPA there was typical biofilm structure at 48 h with adherent microcolonies of live bacteria separated by dark channels, whereas there were few adherent bacteria in the absence of HPA (Fig. 4D). Examination in the x-z plane showed typical three-dimensional biofilm structure (of maximum height 160 μm) only in the presence of HPA.

Pre-exposure to pancreatic amylase promotes interaction with human IECs. To determine if pre-exposure of *C. jejuni* to HPA affects the interaction with IECs, strains grown with or without 100 nM HPA were co-cultured with Caco-2 cells. *C. jejuni* 11168H grown in the presence of HPA showed significant increases in the number of interacting bacteria after 3, 6 and 24 h of co-culture (Fig. 5A). Growth of *C. jejuni* 81-176 in the presence of HPA also resulted in significant increases in interaction with Caco-2 cells (data not shown). In contrast, exposure of the *C. jejuni* 11168H *cj0511* mutant to HPA did not result in increased interaction, whereas the complemented strain did show significant increases (Fig. 5A). Pre-exposure to HPA also resulted in increased invasion by strains 11168H (Fig. 5B) and 81-176 (data not shown). There was no increase in invasion by the *cj0511* mutant with pre-exposure to HPA whereas the complemented strain showed a significant increase (Fig. 5B).

To explore the effect of pre-exposure to HPA on IEC cytokine responses, we measured interleukin-8 (IL-8) secretion from T84 human colon cancer cells in response to bacteria grown with or without HPA. At 24 h, we observed a significant increase in IL-8 secretion in response to infection but growth on HPA had no effect (Fig. 5C). A similar lack of cytokine response to HPA pre-treatment was observed in THP-1 macrophages (data not shown). The effect of infection on transepithelial electrical resistance (TEER) was also investigated. No disruption in tight-junction integrity in response to *C. jejuni* grown on HPA was noted 6 h post-infection (data not shown), but there was a significant increase in translocation of the bacteria pre-exposed to HPA compared to those grown without HPA (Fig. 5D), a difference no longer apparent at 24 h (data not shown).

Pre-exposure to pancreatic amylase results in increased killing of *Galleria mellonella* larvae. *C. jejuni* 11168H, 81-176 and the *cj0511* and *kpsM* mutants were grown with or without HPA, injected into ten *G. mellonella* larvae and the number of dead larvae determined at 24 h (Fig. 6). For both WT strains, a mean of 1 - 2 larvae were killed when the bacteria were grown without HPA compared to means of ~9 when grown with HPA. The increased virulence in response to HPA was lost in the *cj0511* mutant but restored in the complemented strain (Fig. 6), confirming the essential role of Cj0511 in signal detection.

Pre-exposure to pancreatic amylase promotes colonisation of broiler chickens. To determine whether pre-exposure to pancreatic amylase affects the ability of *C. jejuni* to colonise chickens, strain 11168H and the *cj0511* mutant grown with or without HPA were used to infect broiler chickens of 21 days old. At day 4, the WT strain had colonised better than the *cj0511* mutant, but no difference was apparent between treatments (Fig. 7A). By day 7, the number of birds colonised by the WT strain exposed to HPA prior to infection was significantly greater than the number of birds colonised by this strain not exposed to HPA ($p=0.005$) (Fig. 7B). Differences in the numbers of bacteria present between infection groups were seen at day 4 ($p=0.0006$; Kruskal-Wallis test) and day 7 ($p=0.0026$). At day 4 in chickens infected with strains grown in the presence of HPA the WT strain was present in significantly higher numbers in the ileum (13/15 birds infected, median $3.0x10^4$ cfu/g, range 0-$1.5x10^5$ cfu/g) compared to the *cj0511* mutant (5/15 birds infected, median 0 cfu/g, range 0-$1.5x10^4$ cfu/g) (Fig. 7C). Growth on HPA also significantly increased the numbers of the WT strain in the ileum at day 7 (11/15 birds infected, median $2.0x10^4$

375 cfu/g, range 0-3x10^4 cfu/g) compared to the unexposed WT (5/15 birds infected,
376 median 0 cfu/g, range 0-3x10^4 cfu/g), whereas there was no such increase
377 detectable in the *cj0511* mutant (Fig. 7D).
378
379 **Pre-exposure to pancreatic amylase promotes survival in stress environments.**
380 To assess the role of the α-dextran in survival outside the host, we cultured *C. jejuni*
381 11168H in the presence or absence of 100 nM HPA, recovered the bacteria into
382 either broth or PBS without HPA and determined by viable counting the numbers of
383 surviving bacteria at room temperature and at 4ºC in air. In both stress conditions,
384 pre-exposure to HPA prolonged survival suggesting that the α-dextran has a
385 protective effect (Fig. 8).
386
387 **DISCUSSION**
388 Given that *C. jejuni* has been described as a "sugary bug" (45) it is somewhat
389 surprising that until the work described herein, no definitive identification of a
390 polysaccharide involved in biofilm formation had been made. Indeed it has been
391 suggested that DNA is a major component of the *C. jejuni* biofilm matrix (26).
392 However, when *C. jejuni* is freshly isolated from animal or human stools it is highly
393 mucoid (Allan, unpublished observation), suggesting that a polysaccharide matrix is
394 being produced. This led us to hypothesize that a host factor was responsible for
395 inducing the polysaccharide EPM. Such interkingdom signalling between eukaryotes
396 and prokaryotes was first identified over a century ago (46). Over the last decade or
397 so it has been established that interkingdom signalling between bacteria and hosts,
398 via "hormones" is widespread (47). One of the first reports of this was the effect of

399 adrenaline and noradrenaline on *E. coli* (48). There are a limited number of reports
400 on host cell molecules affecting biofilm formation by bacteria. For example, bile salts
401 enhance biofilm formation by *C. jejuni* (26), *Listeria monocytogenes* (49) and *Vibrio*
402 *cholerae* (50). In the case of *Streptococcus pneumonia,* a number of host factors
403 affect biofilm formation and structure including norepinephrine (51), and
404 extracytoplasmic ATP (52). Our work shows for the first time that avian and
405 mammalian pancreatic amylase can act as an interkingdom signalling molecule and
406 enhance *C. jejuni* growth and biofilm formation. It is important to emphasise that a
407 mucoid colony is produced on *Brucella* agar in response to pancreatic amylase. This
408 medium does not contain starch, indicating that the response is directly to the
409 presence of amylase protein and is not dependent on amylase starch degrading
410 activity. This was confirmed in the biofilm assay which was also carried out in
411 medium lacking starch. To our knowledge this is only the second example of a
412 proteinaceous host component that can enhance bacterial biofilm formation, the first
413 being the cytokine interleukin 1β which enhances biofilm formation by
414 *Staphylococcus aureus* (53). Interestingly, *C. rectus* did not appear to detect
415 pancreatic amylase suggesting that the response may be a specific adaptation to
416 host environments. It will be interesting to investigate in future work whether the
417 enteric and oral *Campylobacter* spp. are able to detect the presence of salivary
418 amylase, given its similarity in sequence and structure to pancreatic amylase.
419
420 The polysaccharide secreted by *C. jejuni* was definitively identified as α-dextran and
421 the ease with which it was removed from the bacterial cell surface, by simply
422 washing in saline, indicates that it is a loosely-associated 'slime polysaccharide' as
423 oppose to the capsular polysaccharides that are covalently attached to the cell wall

424 (16, 54). Given that *C. jejuni* is capable of producing complex glycan structures (18),
425 the production of a simple α-dextran is intriguing. Dextrans are produced by oral
426 streptococci and *Leuconostoc* spp. from sucrose using dextransucrases, cell wall-
427 anchored enzymes of the glycoside hydrolase superfamily (56). Searching the
428 translated *C. jejuni* genome sequences with known dextransucrase sequences did
429 not identify homologues, suggesting that dextran synthesis in *C. jejuni* occurs by a
430 novel mechanism. The source of the glucose monomer, whether it is taken up from
431 the environment or synthesized from amino acids, awaits experimental investigation.

432

433 The fact that HPA enhances the growth of *C. jejuni* suggested that it may be used as
434 a nutrient source and Cj0511, predicted as a serine protease of the C-terminal
435 protease (CTP) family and known to be secreted in OMVs (43), seemed a likely
436 candidate for degrading pancreatic amylase. Our data shows that a *cj0511* mutant
437 did not display the response to HPA, a phenotype that was restored in a
438 complemented strain, confirming the role of Cj0511 specifically. However, there was
439 no apparent change in migration of recombinant human amylase on an SDS-PAGE
440 gel following incubation with Cj0511 even though partial casein degradation was
441 apparent in 30 min and complete digestion in 2 h. The recombinant amylase is
442 tagged with 6 histidine residues at the C-terminus and C-terminal proteolysis of even
443 a single amino acid would result in a reduction of ~1 kDa in the molecular weight of
444 the protein which would be apparent on a 16% polyacrylamide gel. In this context, it
445 is important to emphasise that the polyhistidine-tagged human recombinant amylase
446 preparation was active, since we showed it induced biofilm formation in the wild-type
447 strain. Thus, interference by the polyhistidine tag cannot explain why we fail to see

cleavage by r-Cj0511 *in vitro*. Since Cj0511 is N-glycosylated in *C. jejuni* (Young et al., 2002), an absence of glycosylation in r-Cj0511 is another plausible explanation. However, we have shown that a *pglB* mutant (i.e. defective for N-glycosylation; 55) still responds to pancreatic amylase (Jowiya and Allan, unpublished), which discounts this explanation also. Thus we conclude that Cj0511 does not digest pancreatic amylase, at least *in vitro*. Bacterial CTPs are involved in a range of physiological processes. The *Escherichia coli* tail-specific protease processes penicillin binding proteins and regulates cell morphology (57). CTPs with roles in virulence have also been described: for example, CtpA of *Brucella suis* is required for virulence in a mouse infection model (58). A recent study by Karlyshev and co-workers showed that a *cj0511* mutant was severely attenuated in its ability to colonise chickens (42). Since Cj0511 does not appear to degrade pancreatic amylase, we conclude that it is involved at a later stage in the molecular mechanism; elucidation of its precise role requires further research. Previous studies have shown that Cj0511 is expressed in the absence of pancreatic amylase (43) suggesting that its expression is constitutive. It will be interesting in future work to see if Cj0511 levels are increased in the presence of pancreatic amylase.

Having established that pancreatic amylase induces secretion of an α-dextran, we next addressed the biological consequence. Growth on physiological concentrations of HPA resulted in increased biofilm formation in two different WT strains. Moreover, the increase in biofilm formation induced by HPA was dependent on Cj0511. Since the purity of the commercial HPA preparation is not guaranteed, we tested purified recombinant human pancreatic amylase in the biofilm assay and observed an

472 increase in biofilm formation of similar magnitude to that induced by HPA, confirming
473 that the response is to pancreatic amylase specifically. Biofilm formation was also
474 increased in response to an extract of chicken pancreas suggesting that the α-
475 dextran promotes biofilm formation in the intestines of both mammalian and avian
476 hosts.

477 The study next investigated the effect of HPA exposure on the interaction of
478 *C. jejuni* with IECs. Growth on HPA resulted in a significant increase in adhesion and
479 invasion of *C. jejuni* in Caco-2 cells. As the neutrophil chemoattractant IL-8 has a
480 role in the response to *C. jejuni* (59), we studied production by T84 cells as they are
481 more potent cytokine responders than Caco-2 cells (60). Surprisingly, no difference
482 in IL-8 secretion in response to bacteria grown with or without HPA was observed.
483 Pre-exposure to HPA led to a significant increase in bacterial translocation 6 h post-
484 infection, however, without loss of tight junction integrity. Collectively, these data
485 suggest that the α-dextran modulates early host-pathogen interactions by promoting
486 rapid intracellular transfer while avoiding overt host inflammatory responses. It is
487 intriguing to speculate that an α-1,6-dextran evolved as a colonisation factor since it
488 exhibits sufficient similarity to dietary starch to be tolerated by the immune system
489 yet is resistant to the α-1,4-glucan glucanohydrolase activity of pancreatic amylase.

490 In the *G. mellonella* infection model, growth of *C. jejuni* on pancreatic amylase
491 resulted in increased virulence and this is again dependent on Cj0511. *G. mellonella*
492 larvae have been used as a model to study infection by *C. jejuni* (36) and other
493 enteric pathogens as they possess both humoral and cellular immune systems (61).
494 The increased insecticidal activity of *C. jejuni* grown on HPA suggests that biofilm
495 formation may contribute to resistance to these immune mechanisms. It is notable

that the insecticidal activity observed for *C. jejuni* 11168H and 81-176 in the absence of pancreatic amylase was lower than that reported by Champion *et al*. (36) for these strains. We assume that the decreased virulence observed in our study is a reflection of culture on Mueller Hinton agar as oppose to Columbia blood agar which was used by Champion *et al*.

In the laboratory, *C. jejuni* is sensitive to environmental stresses such as drying, chilling, and exposure to atmospheric oxygen yet the bacterium exhibits a remarkable ability to persist in food processing facilities and natural environments (62). We have shown that pre-exposure of *C. jejuni* to pancreatic amylase prolongs survival in atmospheric conditions at ambient and refrigeration temperatures. This demonstrates that the α-dextran, which will be expressed by *C. jejuni* expelled into the environment from a host organism, is stable and may be important for the environmental resilience of the bacterium.

In conclusion, we propose a new model in which *C. jejuni* uses host pancreatic amylase, encountered as it passes the pancreatic duct, as a signal for secretion of an α-dextran which promotes biofilm formation and contributes to colonisation of the mammalian and avian intestine and increases resistance to host immune functions. We have shown that Cj0511, a secreted serine protease, is required for the response to pancreatic amylase although its precise function in the response is currently unknown. The demonstration of a secreted dextran that leads to biofilm formation is an important finding that is expected to enable the design of intervention strategies to reduce the burden of *C. jejuni* in the food chain and ultimately to reduce the incidence of human enteric disease.

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751 Table 1. Bacterial strains and plasmids

752

Bacterial strain	Characteristics	Reference
C. jejuni 11168H	Hyper-motile variant of NCTC 11168; good coloniser of chickens	(63)
C. jejuni 81-176	Clinical isolate from human diarrhoea sample	(64)
C. jejuni 81116	Waterborne human outbreak strain	(65)
C. jejuni X	Clinical isolate from patient with enteritis	(66)
C. jejuni G1	Clinical isolate from patient with Guillain-Barré syndrome	(67)
C. jejuni 11168H *kpsM*::*aphA3*	Mutant in gene encoding ABC transporter involved in capsule assembly; no CPS expression	(68)
C. jejuni 11168H *waaC*::*aphA3*	Mutant in gene encoding heptosyltransferase I; expresses truncated LOS	*Campylobacter* Resource Facility (http://crf.lshtm.ac.uk/index.htm)
C. jejuni 11168H *waaF*::*aphA3*	Mutant in gene encoding heptosyltransferase II: expresses truncated core oligosaccharide	*Campylobacter* Resource Facility (http://crf.lshtm.ac.uk/index.htm)
C. jejuni 11168H *cj0511*::*aphA3*	Mutant in gene encoding secreted carboxyl-terminal protease.	*Campylobacter* Resource Facility (http://crf.lshtm.ac.uk/index.htm)
11168H *cj0511*::*aphA3* pWJ4	Complemented strain containing *cj0511* gene and Cam^r inserted into cj0046	This study
C. rectus NCTC 11489	Type strain, from human periodontal pocket	Public Health England culture collections

E. coli JM109	Cloning host	Promega
E.coli BL21 (DE3)	Expression host	New England Biolabs
Plasmid		
pJMK30	pUC19 carrying *C. coli* Kan^r gene *aphA-3*	(69)
pGEM-T-Easy	Cloning vector enables TA cloning	Promega
pC46fdxA	Complementation vector containing *fdxA* promoter, Cam^r selection marker.	(34)

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755 Table 2. Oligonucleotides

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Name	Sequence (5'-3')	Use
WJ10F	GTCGTCTCACATGTTGAAAACAAAACG	Amplification of *cj0511* for cloning in pC46fdxA (BsmBI site underlined)
WJ10R	GTCGTCTCACATGTTATTGTCCTTGTTTG	Amplification of *cj0511* for cloning in pC46fdxA (BsmBI site underlined)
WJ4	GTAAATTTTTGATTATCAAAATTTACATTATTTAAG	Orientation of *cj0511* with respect to P_{fdxA} in pC46fdxA; anneals in P_{fdxA}
WJ11	GGAACACAGCAGAGCACTTG	Confirmation *of cj0511* complementing strain; anneals in cjs03 RNA coding sequence located 5' to *cj0046*
WJ12	CCTGGAGAAGTATTAGATAGTAGCG	Confirmation of *cj0511* complementing strain; anneals in *cj0053c* located 3' to *cj0046*

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FIG. 1. Exposure to pancreatic amylase promotes growth and results in a large, mucoid colony. A. *C. jejuni* 11168H grown at 37°C for 72 h on BA containing 0.4% charcoal without (left) or with (right) 100 μM HPA. The ruler marks 5 mm intervals. B. *C. jejuni* 11168H growth with shaking (50 rpm) in 5% CO_2 at 37°C in MEM-α with (squares) or without (diamonds) 100 nM HPA. Data reported as means and standard deviations (SD) from three independent experiments. The statistical significance of the difference in growth in the presence and absence of HPA was assessed at two time points (10 and 15 h) using a two-sample t-test, assuming unequal variance; ***p<0.001.

FIG. 2. Physiological concentrations of pancreatic amylase induces α-dextran secretion in *C. jejuni* and the secreted protease, Cj0511 is required. (A) Total extracellular carbohydrate in *C. jejuni* 11168H grown in the presence of increasing concentrations of HPA. Data reported as mean and standard deviation from three experiments (p<0.0001 for the contrast between the three lowest doses and the four highest doses of pancreatic amylase; p<0.0001 for the contrast between 50 mM and 100 nM pancreatic amylase within one-way ANOVA). (B) Total extracellular carbohydrate in different *C. jejuni* strains grown in the presence (black bars) or absence (white bars) of 100 nM HPA. Data reported as mean and standard deviation from three experiments. The *kpsM*, *waaC* and *waaF* mutants were created in strain 11168H. Asterisks indicate statistically significant increases in the presence of HPA determined by a two-sample t test; ***p<0.001, **p<0.01, *p<0.05. (C) ^{1}H-NMR spectra of extracellular material from *C. jejuni* 11168H, a *kpsM* mutant and a commercial α-dextran recorded in D_20 at 7^{o}C using acetone as an internal standard

on a Bruker Avance 400 MHz spectrometer. (D) Total extracellular carbohydrate in *C. jejuni* 11168H (black bars), a *Cj0511* mutant (white bars) and a *Cj0511* complemented strain (grey bars) grown on MHA with increasing concentrations of HPA. A two-way ANOVA showed that the interaction between the bacterial strain and the dose of pancreatic amylase was highly significant ($p<0.0001$). The contrasts comparing the pattern of the response between the wild-type strain and the *Cj0511* mutant and between the mutant and the complemented strain were both statistically significant ($p<0.0001$).

Fig. 3. Proteolysis of casein and pancreatic amylase by r-Cj0511. Coomassie-stained SDS-polyacrylamide gel showing no detectable proteolysis of polyhistidine tagged human pancreatic amylase. Casein served as a positive control.

Fig. 4. Exposure to pancreatic amylase promotes biofilm formation. (A) Biofilm formation at 48 h by *C. jejuni* strains on glass tubes in the presence (black bars) or absence (white bars) of 100 nM HPA. Data reported as means and SD from three independent experiments. Asterisks indicate statistically significant increases in the presence of HPA; ***$p<0.0001$. (B) Biofilm formation at 48 h by *C. jejuni* 11168H on glass tubes in the presence (black bars) or absence (white bars) of 100 nM recombinant human amylase. Data reported as mean +/- SD from three independent experiments. Asterisks indicate statistically significant increases in the presence of pancreatic amylase; ***$p<0.001$. (C) Biofilm formation at 48 h by *C. jejuni* 11168H on glass tubes in the presence (black bars) or absence (white bars) of chicken pancreas extract. Data reported as mean +/- SD from three independent experiments.

807 Asterisks indicate statistically significant increases in the presence of pancreatic
808 amylase; ***p<0.001. (D) CLSM of Live/Dead stained *C. jejuni* 11168H biofilm at 48
809 h. Top panels, confocal images of bacteria in x-y plane, bottom panels, digital
810 images in x-z plane.
811
812 **FIG. 5. Pre-exposure to pancreatic amylase promotes interaction with human**
813 **intestinal epithelial cells.** (A) Interaction with Caco-2 cells by *C. jejuni* 11168H
814 grown in the presence (black bars) or absence (white bars) of 100 nM HPA. Bacteria
815 were co-cultured with Caco-2 cells for 3, 6 and 24 h. (B) Invasion of Caco-2 cells by
816 *C. jejuni* 11168H grown in the presence (black bars) or absence (white bars) of 100
817 nM HPA. (C) Apical IL-8 levels in T84 cells at 24 h in response to *C. jejuni* 11168H,
818 the *Cj0511* mutant and the complemented strain grown in the presence (black bars)
819 and absence (white bars) of 100 nM HPA. (D) Translocation of *C. jejuni* in T84 cells
820 at 6 h. *C. jejuni* 11168H, a *Cj0511* mutant and a complemented strain were grown in
821 the presence (black bars) and absence (white bars) of 100 nM HPA. For (A) to (C),
822 data reported as mean +/- SD from three independent experiments. For (C), analysis
823 by one-way ANOVA showed that there was a significant increase in IL-8 secretion in
824 response to infection ($p<0.05$); the construction of contrasts within ANOVA showed
825 that there was no significant increase in the levels of IL-8 in response to any of the
826 bacterial strains grown with pancreatic amylase compared to the same bacteria
827 grown in its absence ($p>0.05$). For (D), data reported as mean +/- SD from three
828 independent experiments for *C. jejuni* 11168H and the *cj0511* mutant. For the *cj0511*
829 complemented strain, the mean +/- SD from two independent experiments is
830 reported as a result of contamination in the third experiment, thus no statistical
831 analysis is presented. ***$p<0.0001$, *$p<0.05$.

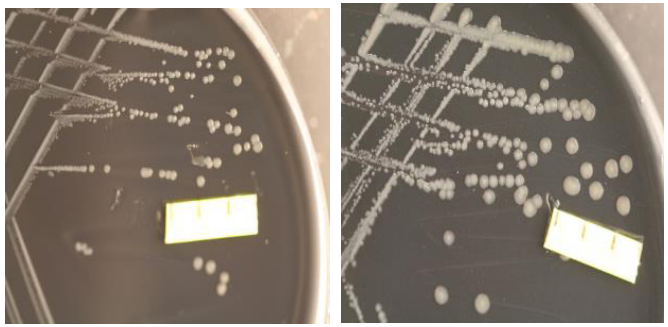
FIG. 6. Pre-exposure to pancreatic amylase promotes infection of *Galleria mellonella* larvae. Killing of *G. mellonella* larvae by *C. jejuni* grown in the presence (black bars) or absence (white bars) of 100 nM HPA. 10 larvae were infected with each strain or with PBS alone. Injection with PBS did not kill any larvae. The *kpsM* mutant and the *cj0511* mutant and complemented strain were derived from *C. jejuni* 11168H. Data reported as mean and SD from three independent experiments. Asterisks indicate statistically significant increases in the presence of HPA; **p=0.001.

FIG. 7. Pre-exposure to pancreatic amylase promotes colonisation of broiler chickens. (A) and (B) number of birds from which *Campylobacter* was isolated from the ileum at 4 d and 7 d post infection, respectively. *C. jejuni* was grown with (black bars) or without HPA (white bars). n=15 birds per group. By day 7, exposure of the WT strain to HPA prior to infection resulted in infection in a greater number of chickens (p=0.005; indicated by bar). (C) and (D) cfu of *Campylobacter* per gram of ileal contents in birds 4 d and 7 d post infection, respectively. Open symbols show strains grown without HPA, closed symbols show strains grown with HPA. Significant differences were seen between groups on days 4 (C; P=0.0006) and 7 (D; P=0.0026) by Kruskal-Wallis test. Bars indicate groups between which differences occur assessed by Dunn's multiple comparison test. n=15 birds per group.

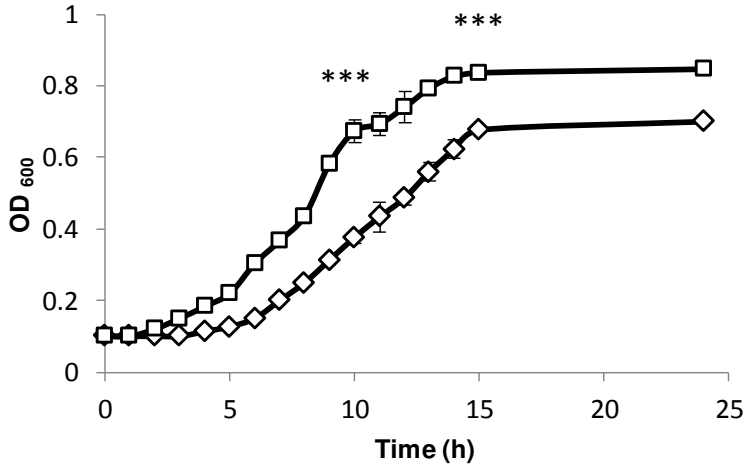
FIG. 8. Pre-exposure to pancreatic amylase promotes survival in stress environments. (A). Survival at 4°C of *C. jejuni* 11168H grown with (squares) or

without (diamonds) HPA. (B). Survival at 20°C in air of *C. jejuni* 11168H grown with (squares) or without (diamonds) HPA. Data reported as means +/- SD from three independent experiments. Statistical significance between the counts in the presence and absence of HPA was assessed using a two-sample t-test, assuming unequal variance; **p<0.01, ***p<0.001.

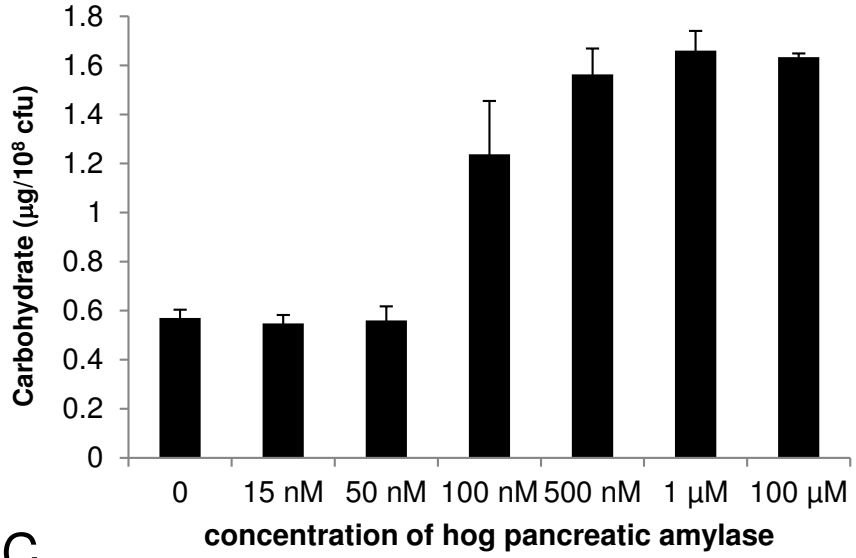
A



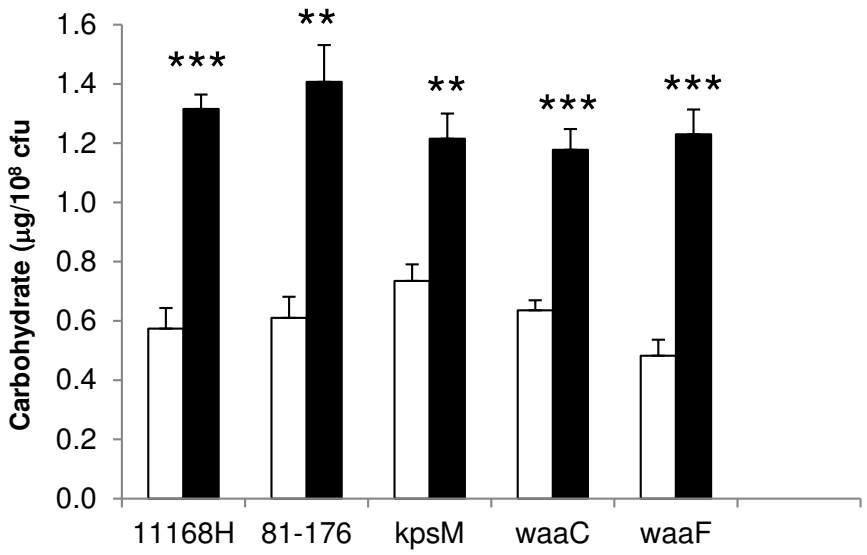
B



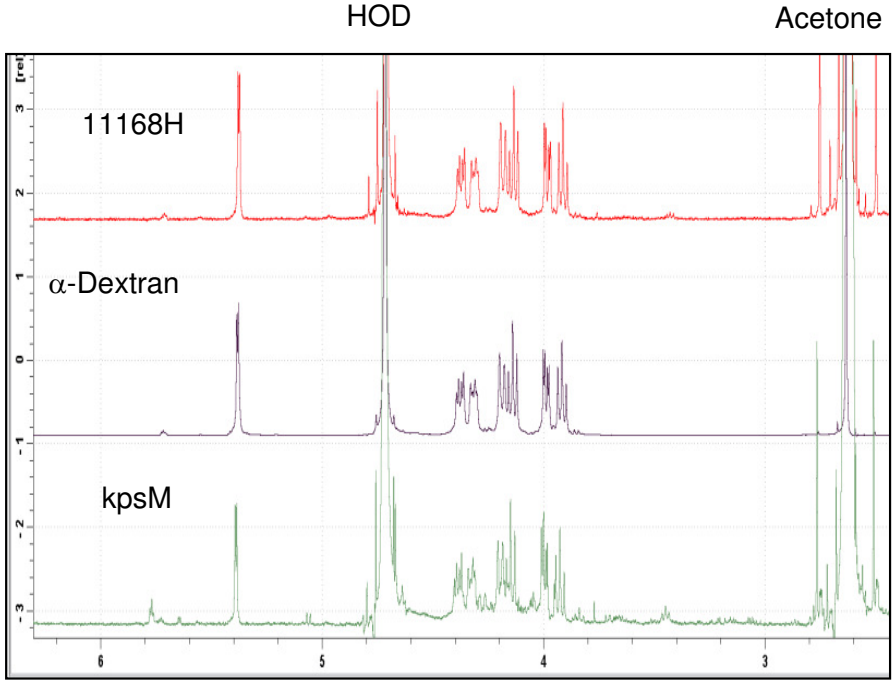
A.


Carbohydrate (µg/10⁸ cfu)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
15 nM
50 nM
100 nM
500 nM
1 µM
100 µM
concentration of hog pancreatic amylase

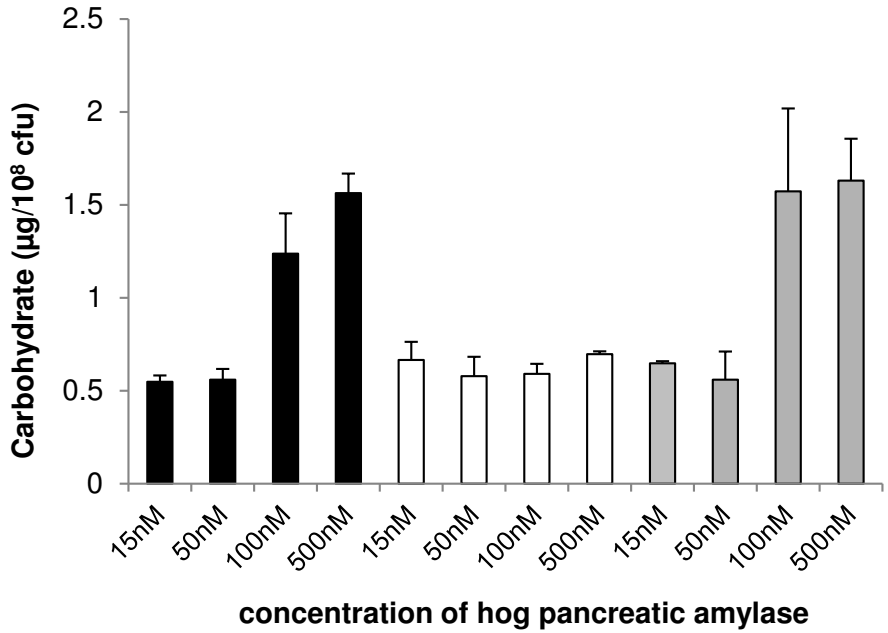
B.

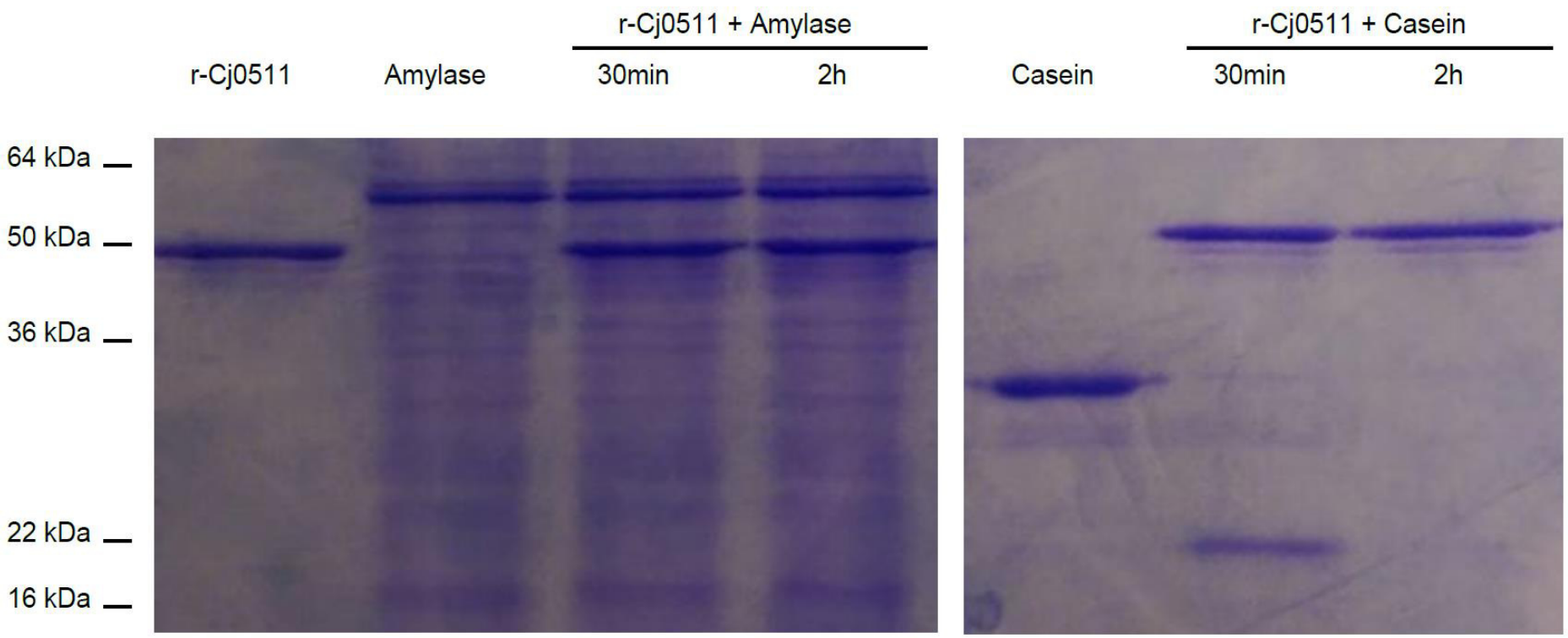

Carbohydrate (µg/10⁸ cfu
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
**
***
***
11168H
81-176
kpsM
waaC
waaF

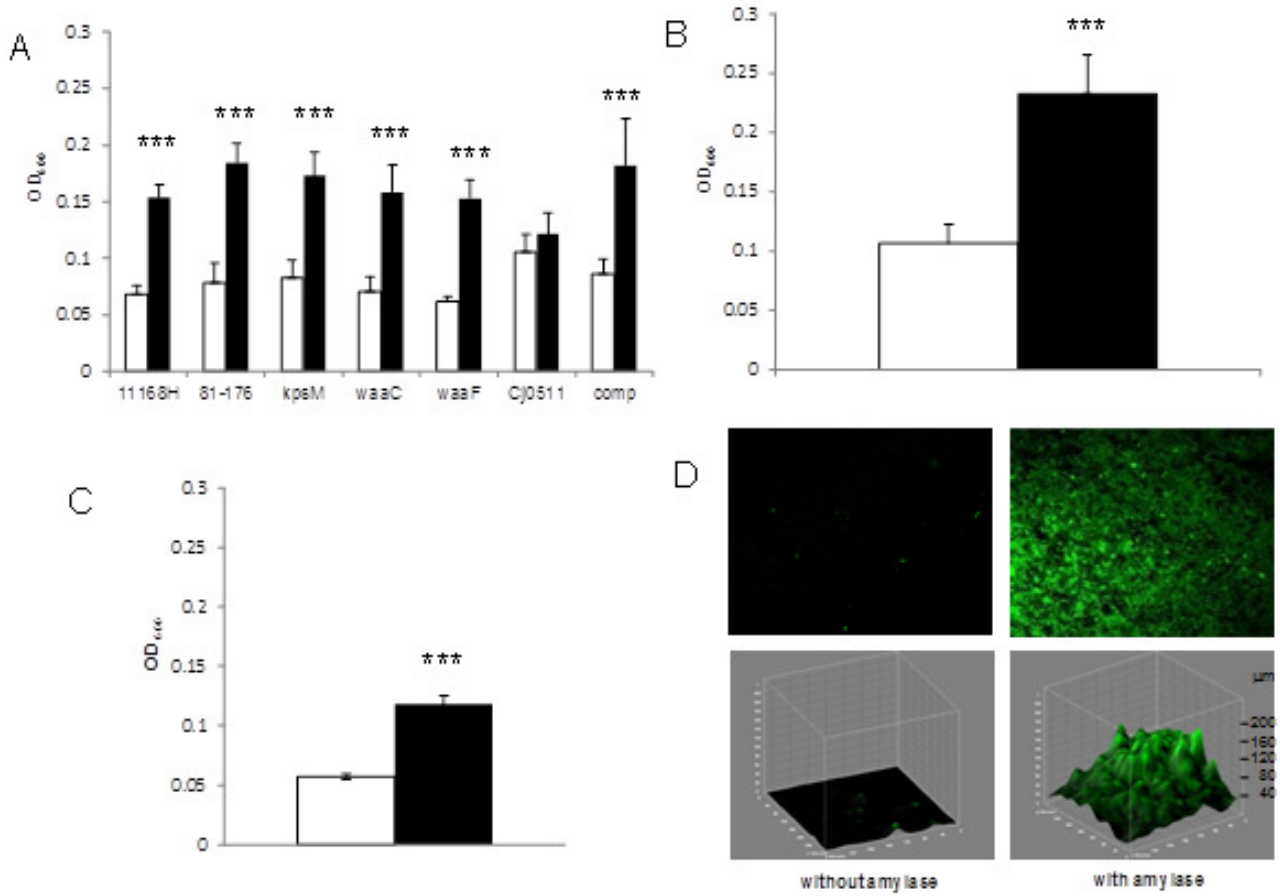
C.

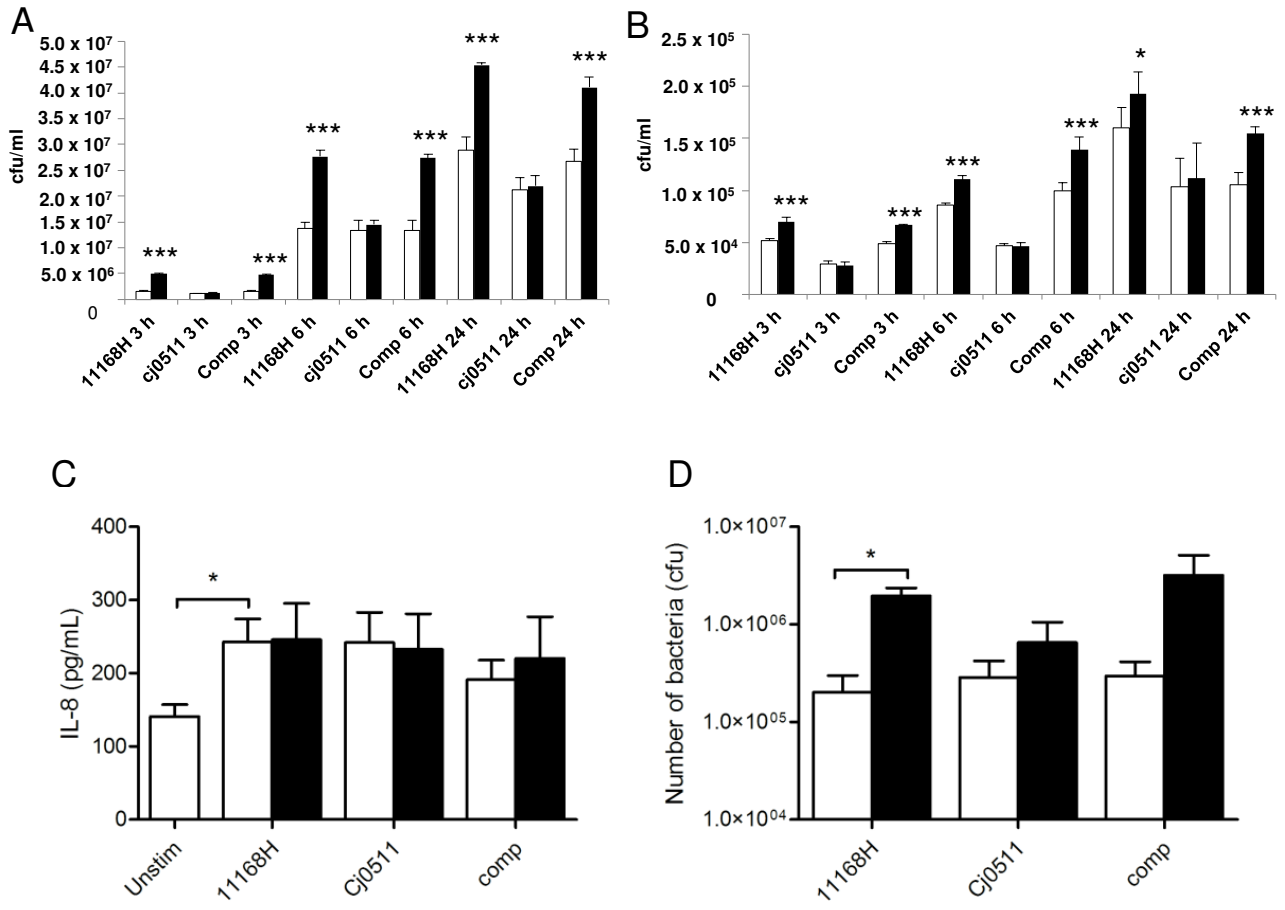

HOD
Acetone
11168H
α-Dextran
kpsM
6
5
4
3

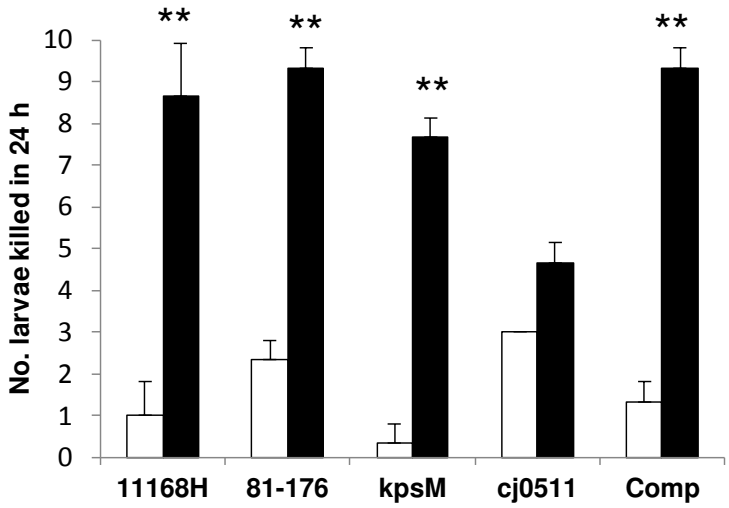
D.

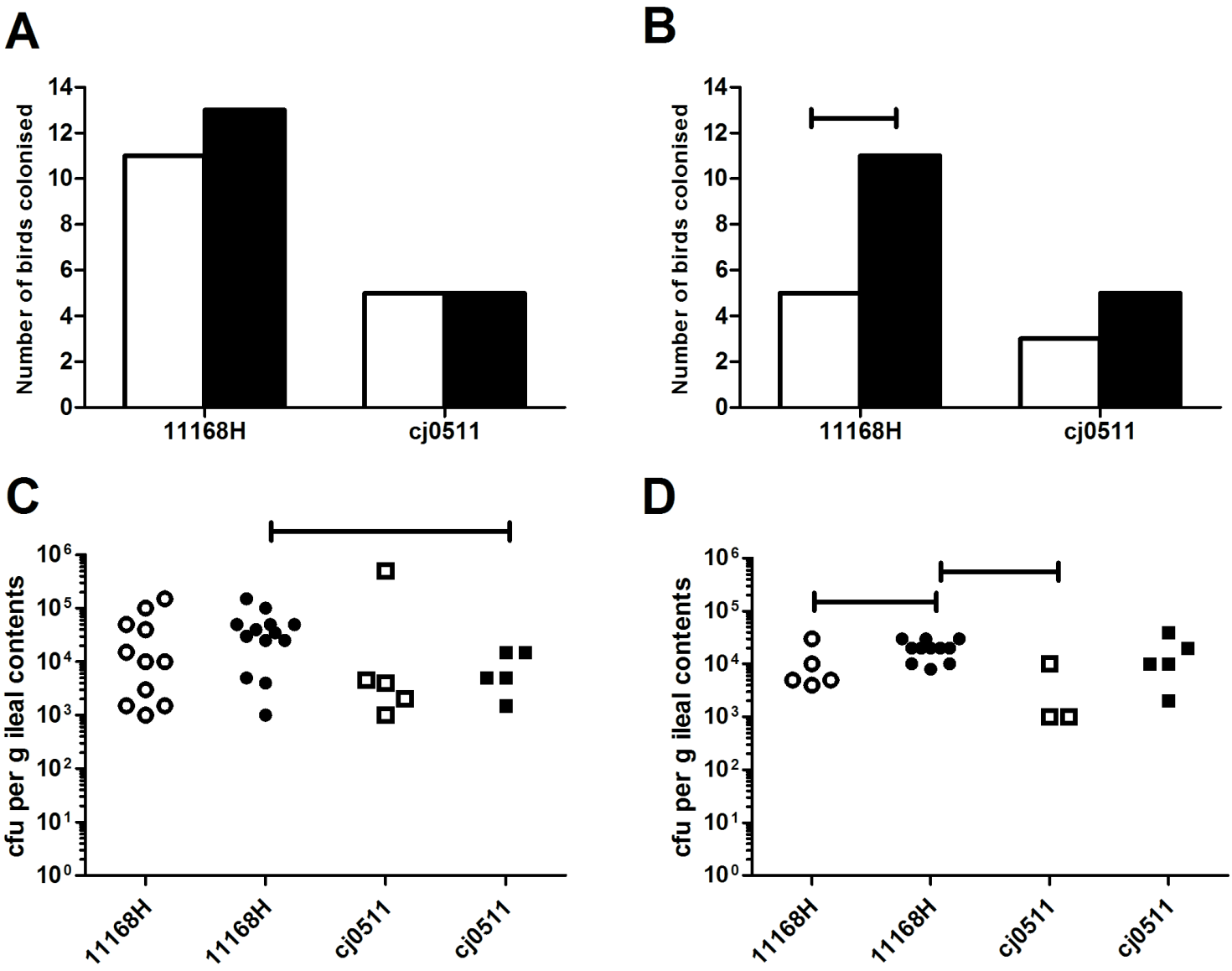

Carbohydrate (µg/10⁸ cfu)
2.5
2
1.5
1
0.5
0
15nM
50nM
100nM
500nM
15nM
50nM
100nM
500nM
15nM
50nM
100nM
500nM
concentration of hog pancreatic amylase


r-Cj0511 + Amylase
r-Cj0511 + Casein
r-Cj0511
Amylase
30min
2h
Casein
30min
2h
64 kDa
50 kDa
36 kDa
22 kDa
16 kDa

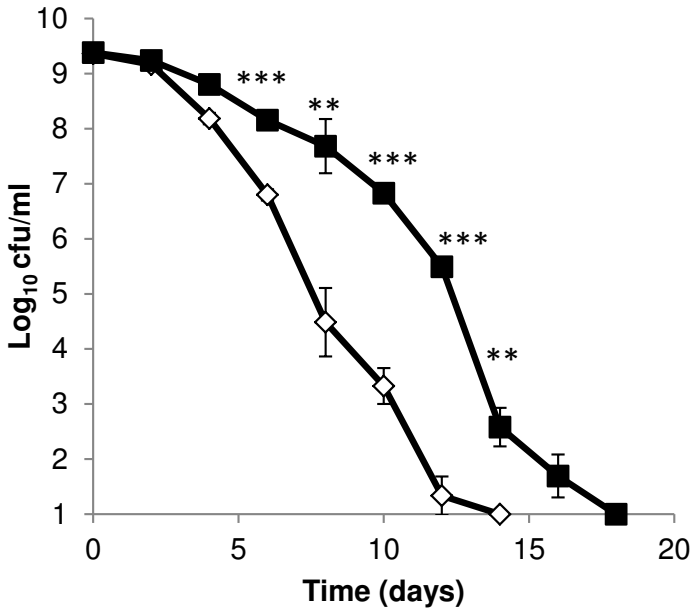

A
B
C
D
OD600
0.3
0.25
0.2
0.15
0.1
0.05
0
***
11168H
81-176
kpsM
waaC
waaF
Cj0511
comp
without amylase
with amylase
µm
200
160
120
80
40


A
cfu/ml
5.0 x 10^7
4.5 x 10^7
4.0 x 10^7
3.5 x 10^7
3.0 x 10^7
2.5 x 10^7
2.0 x 10^7
1.5 x 10^7
1.0 x 10^7
5.0 x 10^6
0
***
11168H 3 h
cj0511 3 h
Comp 3 h
11168H 6 h
cj0511 6 h
Comp 6 h
11168H 24 h
cj0511 24 h
Comp 24 h
B
cfu/ml
2.5 x 10^5
2.0 x 10^5
1.5 x 10^5
1.0 x 10^5
5.0 x 10^4
0
*
C
IL-8 (pg/mL)
400
300
200
100
0
Unstim
11168H
Cj0511
comp
D
Number of bacteria (cfu)
1.0×10^07
1.0×10^06
1.0×10^05
1.0×10^04


**
**
**
**
10
9
8
7
6
5
4
3
2
1
0
No. larvae killed in 24 h
11168H
81-176
kpsM
cj0511
Comp


A
Number of birds colonised
14
12
10
8
6
4
2
0
11168H
cj0511
B
Number of birds colonised
14
12
10
8
6
4
2
0
11168H
cj0511
C
cfu per g ileal contents
10^6
10^5
10^4
10^3
10^2
10^1
10^0
11168H
11168H
cj0511
cj0511
D
cfu per g ileal contents
10^6
10^5
10^4
10^3
10^2
10^1
10^0
11168H
11168H
cj0511
cj0511

A



B