Accepted Manuscript

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PII: S0165-2427(09)00205-0
DOI: doi:10.1016/j.vetimm.2009.06.007
Reference: VETIMM 8093

To appear in: *VETIMM*

Received date: 24-11-2008
Revised date: 10-6-2009
Accepted date: 12-6-2009

Please cite this article as: Shaughnessy, R.G., Meade, K.G., Cahalane, S., Allan, B., Reiman, C., Callanan, J.J., Farrelly, C.O., Innate immune gene expression differentiates the early avian intestinal response between *Salmonella* and *Campylobacter*, *Veterinary Immunology and Immunopathology* (2008), doi:10.1016/j.vetimm.2009.06.007

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**Innate immune gene expression differentiates the early avian intestinal response
between *Salmonella* and *Campylobacter***

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Abstract

Salmonella enterica serovar Typhimurium and *Campylobacter jejuni* are major human pathogens, yet colonise chickens without causing pathology. The aim of this study was to compare intestinal innate immune responses to both bacterial species, in a 4-week-old broiler chicken model. Challenged and control birds were sacrificed and tissue samples taken for histopathology and RNA extraction. No significant clinical or pathological changes were observed in response to infection with either bacterial species. Expression of selected genes involved in pathogen detection and the innate immune response were profiled in caecal tissues by quantitative real-time PCR. *TLR4* and *TLR21* gene expression was transiently increased in response to both bacterial species ($P < 0.05$). Significant increases in *TLR5* and *TLR15* gene expression were detected in response to *S. Typhimurium* but

29 not to *C. jejuni*. Transient increases of proinflammatory cytokine (*IL6* and *IFNG*) and
30 chemokine (*IL8* and *K60*) genes increased as early as 6 hours in response to *S.*
31 Typhimurium. Minimal cytokine gene expression was detected in response to *C.*
32 *jejuni* after 20 hours. *IL8* gene expression however, was significantly increased by 24-
33 fold ($P < 0.01$).

34 The differential expression profiles of innate immune genes in both infection
35 models shed light on the tailored responses of the host immune system to specific
36 microbes. It is further evidence that innate regulation of these responses is an
37 important prerequisite to preventing development of disease.

38

39 Keywords: Chicken; Commensal, Campylobacter; Salmonella; Innate immune gene
40 expression

41

1. Introduction

Constant interaction between multicellular organisms and the microbial world, and concurrent evolution of local innate immune mechanisms has given rise to systems capable of differentiating between benign and harmful microorganisms (Medzhitov, 2007). Bacterial species can be classified as commensal or pathogenic depending on the outcome of these interactions with the underlying host immune system (Hooper & Gordon, 2001). Microbial detection by pattern recognition receptors (PRRs) of the innate immune system induces a signalling cascade resulting in transcriptional changes (Palsson-McDermott & O'Neill, 2007) leading to the production of chemokines, cytokines and other proinflammatory mediators that serve to amplify and regulate cytotoxic activity against potential pathogens (Wick, 2004). These mechanisms have been characterised primarily in mammalian hosts, but are found across the animal kingdom, including in birds (Boyd *et al.*, 2007). While many components of innate immunity are conserved, fundamental differences in local innate immune responses between species may explain why some microorganisms, including *Campylobacter jejuni*, are pathogenic in one species yet a commensal in others.

Toll-like receptors (TLRs), a major family of PRRs, are expressed in chicken intestinal tissues and the local immune cells have been shown to respond to bacterial ligands (Iqbal *et al.*, 2005a). In the caeca of newly hatched chicks, *Salmonella enterica* serovar Typhimurium induces a proinflammatory response (Fasina *et al.*, 2008; Henderson *et al.*, 1999). Influxes of heterophils as well as increases in cytokines and chemokines are evident (Henderson *et al.*, 1999; Withanage *et al.*, 2004) and are thought to contribute to the pathology observed. However, once chicks are more than a few days old, *S. Typhimurium* persistently colonises their intestines in the absence of pathology (Jones *et al.*, 2007), suggesting that maturity of host defences contribute to the lack of clinical signs.

Campylobacter jejuni is another economically important zoonotic bacterial species, and regarded as the leading cause of food-borne human gastroenteritis in the developed world (Hendrixson & DiRita, 2004). *C. jejuni* is primarily an enteric bacterial species (Beery *et al.*, 1988), colonising the caecal crypts of chickens (Newell & Fearnley, 2003). *In vitro*, *C. jejuni* induces proinflammatory cytokines from avian macrophages and kidney cells (Smith *et al.*, 2005), and increased chemokine gene expression has been observed in chicken primary intestinal cells stimulated with the bacterium (Borrmann *et al.*, 2007; Li *et al.*, 2008). However, despite being highly prevalent in the chicken intestine, *C. jejuni* rarely induces disease, regardless of bird age (Van Deun *et al.*, 2007), suggesting possible modulation or evasion of host immune mechanisms.

To date, studies of innate immune gene expression in chicken have focused on investigating the avian intestinal response to pathogenic bacterial species in order to develop strategies to control bacterial colonisation. Under non-pathogenic conditions, the role of the innate immune response to both of these bacterial species is poorly understood, especially whether such responses if present, impact on the levels of bacterial colonisation. As co-evolutionary histories of host and microbial species are likely to differ, we hypothesized that underlying innate immune gene expression would differ in response to both bacterial species and might reveal differential mechanisms for establishing the commensal state. We therefore carried out *in vivo* infection models with *S. Typhimurium* and *C. jejuni* to compare the local innate immune response in the caecums of challenged birds.

2. Materials and Methods

2.1 Bacterial strain preparation

Salmonella enterica serovar Typhimurium strain SL1344 was cultivated in modified N-minimal medium containing 5 mM KCL, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 100 mM Tris-HCL (Ph 7.0) 38 mM glycerol, 0.1% Casamino Acids, 24 mM MgCl₂, 337 μ m PO₄³ at 37°C with shaking at 200 rpm until an optical density at 600 nm of 0.7 was reached. The cells were harvested by centrifugation and resuspended in 0.85% NaCl to one-tenth the original volume. The number of viable bacteria present in the challenge was determined by viable cell counts on Luria-Bertani (LB) agar plates and calculated to be 1×10^{10} /ml. *Campylobacter jejuni* strain NCTC11168 v1 was grown on Mueller Hinton (MH) agar plates. The cells were resuspended in saline and viable bacterial numbers were determined on MH agar plates (2.5×10^8 /ml).

2.2 Experimental challenge

Both *S. Typhimurium* and *C. jejuni* infection models were performed as previously described (Meade *et al.*, 2008). For the analysis of the caecal immune response to both *C. jejuni* and *S. Typhimurium* challenge, 78 4-week-old birds were used from this study. 36 birds represented either mock-challenged or *S. Typhimurium*-challenged birds at each of three timepoints: 6 hours, 20 hours and 48 hours (n = 6 birds per timepoint). Similarly, 42 birds were used for analysis of the caecal immune response to *C. jejuni* challenge, representing either mock-challenged or *C. jejuni*-challenged birds at the same timepoints (n = 7 birds per timepoint).

2.3 Microbiology

All birds were examined for the presence of either *S. Typhimurium* or *C. jejuni* using cloacal swabs at the beginning of each experiment. To determine the presence and level of intestinal colonisation during the experiment, caecal samples

were cultured using serial dilutions. To detect *S. Typhimurium*, the samples were cultured on Brilliant Green Agar at 37°C for 20 hours. To detect *C. jejuni*, the samples were plated on Karmali agar and incubated microaerophilically at 42°C for 48 hours.

2.4 Histopathology

At post mortem, sections of caecum were sampled and preserved in liquid nitrogen and subsequently stored at -80°C. Prior to histopathological evaluations, caecal samples were brought to room temperature before being placed in formalin for routine fixation. Tissue from 2/3 animals per timepoint was trimmed, paraffin-embedded, sectioned at 5-8 µm thickness and stained with haematoxylin and eosin (H&E).

2.5 RNA extraction, cDNA synthesis & real time quantitative RT-PCR

Total RNA was extracted from tissue samples using a combination of (TriReagent®, the MELT™ Total Nucleic Acid Isolation System (Ambion). All samples were DNase treated. RNA yield and quality was then assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Two µg of total RNA from each sample was reverse transcribed into cDNA with oligo-dT primers using SuperScript III® first strand synthesis SuperMix kit according to the manufacturer's instructions (Invitrogen Ltd, Paisley, UK). A total of 40ng cDNA, quantified using a NanoDrop® ND-1000 spectrophotometer, was subsequently used for each real time qRT-PCR reaction. Primers for real time qRT-PCR were designed, intron-spanning where possible, using Primer3 software (Rozen & Skaletsky, 2000) and commercially synthesised (Invitrogen Ltd, Paisley, UK). Primer sets are described in Supplementary table 1. Each reaction was carried out in duplicate, in a total volume of 25 µl with 2 µl of cDNA (20 ng/µl), 12.5 µl PCR master mix (Stratagene Corp, La Jolla, CA) and 10.5 µl primer/H₂O. Optimal primer concentrations were determined by titrating 100, 300 and 900 nM final concentrations and dissociation curves were examined for the

151 presence of a single product. Real time qRT-PCR was performed using a MX3000P®
152 quantitative PCR system (Stratagene Corp, La Jolla, CA) using the following cycling
153 parameters: 95°C for 30 s. 60°C for 1 min and 72°C for 30 s followed by amplicon
154 dissociation. All gene amplifications were normalised to Ribosomal protein L7
155 (*RPL7*) which was selected as the most stably expressed gene across samples in both
156 challenge models from a panel of potential normalisers (*ACTB*, *GAPDH*, *LDHA* and
157 *RPL7*) using GeNorm version 3.4 (Vandesompele *et al.*, 2002).

159 2.6 Data analysis

160 Data analysis was carried out using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen,
161 2001). Analyses were carried out within, and not between infection models to prevent
162 the introduction of stochastic error due to the variation between individual animals.
163 Statistical analyses were performed using non-parametric Mann-Whitney U test as
164 implemented in version 5.01 of StatView (SAS Institute Inc. Cary, NC, USA).

3. Results

3.1 Caecal colonisation

Levels of *S. Typhimurium* and *C. jejuni* colonisation were determined in a range of tissues and have been previously described (Meade *et al.*, 2008). Two challenged chickens (one infected with *S. Typhimurium* and one with *C. jejuni*) were culture negative across all tissues and were excluded from subsequent gene expression analysis. Specific to the caecum, persistent colonisation was detected in response to both bacterial challenges across the 48-hour timecourse. High levels of *S. Typhimurium* colonised the caecums of challenged birds at all three timepoints. A mean of 2.12×10^7 cfu/g was detected at 6 hours, a mean of 1.29×10^7 cfu/g at 20 hours, and a mean of 1.15×10^7 cfu/g at 48 hours. Levels of *C. jejuni* colonisation in the caecum were higher than with the *S. Typhimurium* challenge across the timecourse: 6 hours (a mean of 2.35×10^8 cfu/g; 10 fold higher), 20 hours (a mean of 2.25×10^{11} cfu/g; 10,000 fold higher) and 48 hours (a mean of 8.58×10^{11} cfu/g; 10,000 fold higher). Control animals were negative for both bacterial species. Results demonstrate consistent bacterial colonisation of the caecum in both challenge models (Meade *et al.*, 2008).

3.2 Histopathological analysis

Histopathological analysis of caecal samples obtained throughout the time course of challenge revealed only slight differences between both infection models that were not interval dependent. Regarding inflammation, there were no notable differences between both bacterial challenges and their uninfected controls. Crypt architecture was similar between controls and infected in both challenge models. Regions of gut associated lymphoid tissue (GALT) were prominent in many of the samples selected for examination and consequently, varying degrees of lymphocyte infiltration of the lamina propria were observed but not thought to be in response to

either of the bacterial species. Only subtle increases in heterophils were detected in response to *S. Typhimurium* at 6, 20 and 48 hours post-challenge. Heterophil migration was not detected in response to *C. jejuni* at any time point (data not shown).

3.3 Gene expression of pan-leukocyte marker *CD45*

CD45, also known as protein tyrosine phosphatase receptor type C, is a cell surface marker on all immune cells of haematopoietic origin. Measuring the mRNA expression of this marker is one way of determining cellular changes at the site of caecal infection (Fig. 1). There was a significant but transient increase of *CD45* gene expression to both *S. Typhimurium* (2.8 fold; $P=0.004$) and *C. jejuni* (2.6 fold; $P=0.025$) at 6 hours post challenge. In contrast, there was a significant decrease in *CD45* (-2.2 fold; $P=0.004$) in response to *S. Typhimurium* 20 hours post-challenge but *CD45* was not differentially expressed in response to *C. jejuni* at this time point. Profiling *CD45* gene expression 48 hours post-challenge did not reveal any significant changes in response to either bacterial species (Fig. 1).

3.4 Bacterial induced TLR gene expression profiling

We compared TLR gene expression changes in response to both bacterial challenges (Fig. 2). Of the 4 TLRs examined by qRT-PCR, all 4 genes were significantly differentially expressed in one or other infection model ($P < 0.050$).

TLR4, which detects LPS, was transiently increased by 2.3 fold ($P=0.037$) at 6 hours post-challenge with *S. Typhimurium* followed by a significant reduction after 20 hours (-3.4 fold; $P=0.004$), and not differentially expressed after 48 hours (Fig. 2A). Increased *TLR4* (4.0 fold; $P=0.025$) gene expression was detected in response to *C. jejuni* after 6 hours and was not differentially expressed for the remainder of the timecourse (Fig. 2B).

TLR5, which detects flagellin, was also transiently increased in response to *S. Typhimurium* by 1.9 fold ($P=0.053$) after 6 hours, decreased significantly at 20 hours (-1.3 fold; $P=0.053$), and was increased again by 48 hours (2.2 fold; $P=0.055$) post-challenge (Fig. 2A). *TLR5* was not differentially expressed in response to *C. jejuni* across the timecourse.

TLR15 gene expression was decreased in response to *S. Typhimurium* at 20 hours (-1.2 fold; $P=0.055$) and increased at 48 hours (6.2 fold; $P=0.004$) post-challenge (Fig. 2A). In contrast, *TLR15* was not differentially expressed in response to *C. jejuni* across the timecourse.

TLR21 gene expression was increased in response to both *S. Typhimurium* (5.2 fold; $P=0.004$) and *C. jejuni* (3.5 fold; $P=0.025$) at 6 hours post-challenge. Differential *TLR21* gene expression was not evident for the remainder of timecourse to either infection (Fig. 2A&B).

3.5 Comparative chemokine and cytokine gene expression profiling

Profiling of cytokines and chemokine gene expression was performed to detect differences in both infection models, and characterise the predominant type of immune response across the timecourse. *IFNG* gene expression was transiently increased at 6 hours (4.1 fold; $P=0.055$), not differentially expressed at 20 hours, and significantly increased at 48 hours (6.0 fold; $P=0.007$) post-challenge with *S. Typhimurium* (Fig. 3A). In contrast, *IFNG* was not differentially expressed in response to *C. jejuni* at any timepoint across the timecourse.

In response to *S. Typhimurium*, differential gene expression of *IL1 β* was first detected at 20 hours with an increase of 2.3 fold ($P=0.050$), followed by a further increase of 7.4 fold ($P=0.004$) at 48 hours post-challenge (Fig. 3A). Similarly, increased *IL1 β* gene expression was detected after both 20 hours (5.2 fold; $P=0.005$) and 48 hours (3.9 fold; $P=0.025$) in response to *C. jejuni* (Fig. 3B).

246 *IL6* gene expression was significantly increased in response to *S.*
 247 Typhimurium at 6 hours (11.7 fold; $P=0.004$), 20 hours (1.9 fold; $P=0.054$) and 48
 248 hours (19.4 fold; $P=0.004$) post-challenge (Fig. 3A). Differential gene expression of
 249 *IL6* in response to *C. jejuni* was evident only after 48 hours (4.4 fold; $P=0.025$) post-
 250 challenge (Fig. 3B).

251 Increased *TGFB4* gene expression in response to *S. Typhimurium* at 6 hours
 252 (1.6 fold; $P=0.004$), 20 hours (2.4 fold; $P=0.055$) and 48 hours (1.9 fold; $P=0.003$)
 253 post challenge (Fig. 3A). In contrast *TGFB4* gene expression was not differentially
 254 expressed in response to *C. jejuni* throughout the timecourse.

255 Gene expression of the chemokine *IL8* was significantly increased in response
 256 to *S. Typhimurium* 6 hours (5.1 fold; $P=0.004$), not differentially expressed at 20
 257 hours, and again increased at 48 hours (11.5 fold; $P=0.055$) post-challenge (Fig. 4A).
 258 There was a significant decrease of *IL8* (-2.9 fold; $P=0.030$) gene expression and in
 259 response to *C. jejuni* after 6 hours, followed by a substantial increase at 20 hours (24.3
 260 fold; $P=0.002$) and a reduction at 48 hours (3.3 fold; $P=0.002$) post-challenge (Fig.
 261 4B).

262 Increased expression of another chemokine, *K60* was also evident in response
 263 to *S. Typhimurium* after 6 hours (2.1 fold; $P=0.004$), 20 hours (4.3 fold; $P=0.004$) and
 264 48 hours (4.1 fold; $P=0.043$) post-challenge (Fig. 4A). In response to *C. jejuni*, there
 265 was a transient decrease of *K60* gene expression at 6 hours (-4.7 fold; $P=0.024$)
 266 followed by a transient increase at 20 hours (6.6 fold; $P=0.002$) post challenge (Fig.
 267 4B).

268

269 3.6 *C. jejuni* induced chemokine gene expression in the caecal tonsil

270 To determine if the source of the elevated *IL8* chemokine gene expression was
 271 from the lymphoid tissue in the caecal tonsil, *IL8* and *K60* were profiled, in response
 272 to *C. jejuni* (Fig. 5). Increased *IL8* gene expression was detected 6 hours (2.3 fold;

273 $P=0.025$), 20 hours (4.5 fold; $P=0.002$) and 48 hours (7.4 fold; $P=0.025$) post-
274 challenge. *K60* mRNA was significantly increased only at 48 hours post-challenge
275 (2.3 fold; $P=0.025$).
276

4. Discussion

To date, studies of innate immune gene expression in the chicken have focused on the avian intestinal response to pathogenic bacterial species. However, two zoonotically important microorganisms, *S. Typhimurium* and *C. jejuni*, persist in the intestines of birds in the absence of clinical signs of disease. Under non-pathogenic conditions, the role of the immune response to both of these bacterial species is only partially known, especially whether such responses influence the level of bacterial colonisation. *S. Typhimurium* is capable of inducing immune mediated pathology in only newly hatched chicks (Henderson *et al.*, 1999) and not in 1-week-old chicks (Jones *et al.*, 2007), indicating that maturity of host defences may be important. In contrast, *C. jejuni* resides as a commensal in chickens, regardless of age (Young *et al.*, 2007), suggesting possible modulation or evasion of immune mechanisms. We therefore hypothesised that underlying differences in innate immune gene expression in response to both bacterial species may reveal mechanisms for establishing the commensal state.

As expected, both bacterial species persistently colonised the caecum of challenged birds across the 48-hour timecourse. In comparison to *S. Typhimurium* colonisation, *C. jejuni* colonisation levels were higher most notably at 20 hours and 48 hours post-challenge (10,000 fold higher), indicating *C. jejuni*'s ability to thrive in the caecal environment. Despite persistent bacterial colonisation, clinical signs of disease were not observed in either infection model. Previous studies have shown marked heterophil infiltration to the lamina propria in chicks following *S. Typhimurium* challenge (Berndt *et al.*, 2007; Henderson *et al.*, 1999). Only subtle heterophil infiltration to the caecum was detected in this study of 4 week-old birds in response to *S. Typhimurium*. Heterophil influx was clearly absent from the *C. jejuni* challenge, in agreement with recent literature (Hendrixson & DiRita, 2004). To further characterise cellular changes in response to infection, but at the molecular level, gene expression

of *CD45* was also examined. *CD45* is a transmembrane receptor expressed on cells of haematopoietic origin including heterophils. We observed only minor increases of *CD45* gene expression at 6 hours in response to both bacterial challenges. *CD45* is upregulated on activated inflammatory cells (Hermiston *et al.*, 2003) and therefore such increases may represent transient activation of residential phagocytes of the lamina propria in response to the high bacterial challenges. The lack of increased *CD45* gene expression for the remainder of the timecourse to either infection suggests an absence of immune cell infiltration or activation and is in agreement with the histological observations.

We next quantified expression of genes involved with microbial detection and activation of subsequent innate immune responses. As early as 6 hours post-challenge, expression of *TLR4* and *TLR21* was transiently increased in caecal tissue in response to both bacterial challenges. Chicken *TLR4* recognises LPS and has been associated with *Salmonella* infection (Kogut *et al.*, 2005; Leveque *et al.*, 2003) while a ligand for *TLR21* has yet to be identified. Interestingly, increased *TLR5* gene expression was detected only in response to *S. Typhimurium* during the timecourse (6 hours and 48 hours). *TLR5* activation by flagellin from *S. Typhimurium* has been demonstrated to induce increased *IL1 β* mRNA from chicken cells (Iqbal *et al.*, 2005) but *C. jejuni*'s flagellin evades detection by human *TLR5* (Andersen-Nissen *et al.*, 2005; Johanesen & Dwinell, 2006). The lack of increased *TLR5* gene expression in response to *C. jejuni* suggests a similar mechanism of evasion in the avian host. Similarly, *TLR15* was increased in response to *S. Typhimurium* but not in response to *C. jejuni*. Higgs *et al.* have previously shown an increase in caecal *TLR15* mRNA in response to heat-killed *S. Typhimurium* (Higgs *et al.*, 2006). Unchanged expression of *TLR5* and *TLR15* genes in response to *C. jejuni* throughout the timecourse highlights key differences in the recognition of both bacterial challenges.

Previous studies showed that *S. Typhimurium* induces expression of a range of proinflammatory cytokines and chemokines in newly hatched chicks accompanied by clinical signs of disease (Withanage *et al.*, 2004; Withanage *et al.*, 2005). In our study, in the absence of pathology, we still found increased proinflammatory cytokine and chemokine gene expression in response to *S. Typhimurium* post-challenge in the caecum of challenged chickens. *IFNG*, *IL6* and *IL8* gene expression changes followed a similar pattern: transient increases at 6 hours, no increase at 20 hours, and significant increased expression evident again at 48 hours. Intermittent invasion of the caecal epithelium and subsequent encounters with underlying phagocytes may account for these observations. Significant increases of *IL1B*, *K60* and *TGFB4* were also evident in response to *S. Typhimurium*.

In contrast, in response to *C. jejuni*, increased expression of proinflammatory genes was not detected until 20 hours post-challenge. Therefore, the delayed response may be due to *C. jejuni* invading the caecal epithelium at a slower rate than *S. Typhimurium*. Based on mammalian studies, it is likely that the basolateral surfaces of the caecal epithelium have greater TLR expression and are thus more inductive of proinflammatory responses. Invasion is required to encounter the basolateral surface and the immunopotent phagocytes of the lamina propria. Therefore, the delayed proinflammatory response may be due to *C. jejuni* invading the caecal epithelium at a slower rate than *S. Typhimurium*. While only minor increases in *IL1B* and *K60* were evident, they were accompanied by high *IL8* gene expression. A recent study has also shown increased expression of *IL8* and *K60* genes accompanied by minimal changes in cytokine gene expression in intestinal tissues of both newly hatched and 2 week old chickens challenged with *C. jejuni* (Smith *et al.*, 2008). At 48 hours post-challenge, *IL8* gene expression remained elevated although to a much lesser extent. High *IL8* gene expression followed by reduced expression might be a result of *C. jejuni* temporarily invading the epithelium and then returning to the mucus layer as the

microorganism multiplies in mucus and not epithelial cells (Van Deun *et al.*, 2007).
As *C. jejuni* colonisation in the mucosae would become more established, epithelial
invasion would be less likely.

The caecal tonsils are a defined aggregate of lymphoid tissue, rich in
lymphocyte and phagocytic cells, inductive of proinflammatory gene expression in
response to bacterial stimulus (Haghighi *et al.*, 2008). We observed significantly
increased *IL8* gene expression at 6 hours, 20 hours and 48 hours post-challenge.
Increased *K60* gene expression was also evident at 48 hours post *C. jejuni*-challenge
in the caecal tonsil. These results suggest that the caecal tonsils are an important site
for intestinal IL-8 production in response to *C. jejuni*. Chicken IL-8 has previously
been shown to attract both heterophils and monocytes (Barker *et al.*, 1993; Poh *et al.*,
2008). Yet, our histological data in combination with unchanged *CD45* gene
expression in the presence of high *IL8* gene expression indicates no inflammatory cell
influx in response to *C. jejuni*. These results suggest IL-8 is post-transcriptionally
modified or perhaps that *C. jejuni* has developed mechanisms to interrupt the
chemotactic properties of this chemokine.

The results from this study provide some intriguing insights into the nature of
the intestinal immune gene expression patterns that are integral to the host response to
both bacterial species. Furthermore, it identifies some key molecules that characterise
and differentiate the immune response to *C. jejuni*. Future work will concentrate on
the elucidation of pathways regulating the *IL8* gene expression detected in the caecum
and investigate the mechanisms that may suppress the chemotactic and pathological
effects of this chemokine. Uncovering the specific regulatory mechanisms activated in
response to high-level *C. jejuni* colonisation may shed light on the role of the host
innate immune response in the development of commensal tolerance in the chicken.

383 **Acknowledgements**

384 This work was supported by grants from both the Irish Department of
385 Agriculture and Food (Food Institutional Research Measure) funding (06RDD486 -
386 COF) and Advancing Canadian Agriculture and Agri-Food Program (NA0133 - BA).

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Figure captions

Fig. 1. Comparative *CD45* (a marker for proinflammatory cell migration) gene expression profiles in response to both *S. Typhimurium* (n=6) and *C. jejuni* (n=7) in the caecum at 6 hours, 20 hours and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted * $P < 0.05$, ** $P < 0.01$).

Fig. 2. Comparative TLR gene expression profiles in response to both (A) *S. Typhimurium* (n=6) and (B) *C. jejuni* (n=7) in the caecum at 6 hours, 20 hours and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted * $P < 0.05$, ** $P < 0.01$).

Fig. 3. Comparative cytokine gene expression profiles in response to both (A) *S. Typhimurium* (n=6) and (B) *C. jejuni* (n=7) in the caecum at 6 hours, 20 hours and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted * $P < 0.05$, ** $P < 0.01$).

Fig. 4. Comparative caecal chemokine gene expression profiles for (A) *K60* and (B) *IL8*, in response to both *S. Typhimurium* (n=6) and *C. jejuni* (n=7) 6 hours, 20 hours

and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls. Statistical analysis was carried out separately for each infection model to compare challenged and control birds (significantly differentially expressed genes are highlighted $*P < 0.05$, $**P < 0.01$).

Fig. 5. *IL8* and *K60* chemokine gene expression profiles in the caecal tonsil in response to *C. jejuni* (n=7) at 6 hours, 20 hours and 48 hours post-challenge. The horizontal lines within each box plot represents the median fold changes in gene expression in infected chickens relative to the mock-infected controls (significantly differentially expressed genes are highlighted $*P < 0.05$, $**P < 0.01$).

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Supplementary table 1. Real time qRT-PCR primer sequences, Genbank accession number, amplicon sizes and optimum primer concentrations for all genes analysed.

Gene Symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number	Amplicon Size (bp)	Primer Conc. (nm)
<i>CD45</i>	TATTCTGGTGTCTTGATTGTTGTG	CTGCTACAAGGCTGATGACTTCA	NM_204417.1	120	300
<i>TLR4</i>	AGTCTGAAATTGCTGAGCTCAAAT	GCGACGTTAAGCCATGGAAG	NM_001030693.1	190	300
<i>TLR5</i>	CCTTGTGCTTTGAGGAACGAGA	CACCCATCTTTGAGAACTGCC	NM_001024586	124	300
<i>TLR15</i>	GTTCTCTCTCCCAGTTTTGTAAATAGC	GTGGTTTCATTGGTTGTTTTAGGAC	NM_001037835	262	300
<i>TLR21</i>	TGCCCCTCCCACTGCTGTCCACT	AAAGGTGCCTTGACATCCT	NM_001030558.1	112	300
<i>IL1β</i>	TCGACATCAACCAGAAGTGC	GAGCTTGTAGCCCTTGATGC	DQ393267	185	900
<i>IL6</i>	AGAAATCCCTCCTCGCCAAT	AAATAGCGAACGGCCCTCA	NM_204628.1	120	900
<i>IFNG</i>	AGTCCCGATGAACGACTTG	ATCTCCTCTGAGACTGGCTCCTT	NM_205149	120	300
<i>IL8</i>	GTTTCAGCTGCTCTGTCGAA	GGCGTCAGCTTCACATCTTGA	DQ393272	125	900
<i>K6O</i>	GCCAGTGCATAGAGACCCATTC	TGCCATCTTTCAGAGTAGCTATGACT	NM_205018	119	300
<i>RPL7</i>	ACATGTACAGACAGGAGATCCGCA	AAGCTGTAACACCTTCCGGACCTT	NM_001006345	146	300
<i>ACTB</i>	CCACAGCTGCCTCTAGCTCTTC	AGGACTCCATACCCAAGAAAGATG	NM_205518	132	900
<i>LDHA</i>	CCTGAAGGCTCTTCATCCAG	TTCAGCTAGATCTGCCACAGAA	NM_205284	154	900
<i>GAPDH</i>	AGCTTACTGGAATGGCTTTCCG	ATCAGCAGCAGCCTTCACTACC	NM_204305	122	900
<i>TGFB4</i>	GGATGGACCCGATGA	CGTTGAACACGAAGAAGATGCT	M31160	119	900

Supplementary table 2. Real time qRT-PCR median fold change in the caecums of *S. Typhimurium* infected birds (n = 6) relative to mock-infected controls at 6 hours, 20 hours and 48 hours post-challenge. *P* values were determined using Mann-Whitney U test.

Gene Symbol	6 hours post-infection		20 hours post-infection		48 hours post-infection	
	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
<i>CD45</i>	2.8	0.004	-2.2	0.004	0	>0.999
<i>TLR4</i>	2.3	0.037	-3.4	0.004	0	>0.999
<i>TLR5</i>	1.9	0.053	-1.3	0.053	2.2	0.055
<i>TLR15</i>	2.5	0.200	-1.2	0.055	6.2	0.004
<i>TLR21</i>	5.2	0.004	-1.1	0.054	1.6	0.337
<i>IL1β</i>	2.3	0.335	2.3	0.050	7.4	0.004
<i>IL6</i>	11.7	0.004	1.9	0.054	19.4	0.004
<i>IFNG</i>	4.1	0.055	-2.1	0.337	6.0	0.007
<i>TGFB4</i>	1.6	0.004	2.4	0.055	1.9	0.003
<i>IL8</i>	5.1	0.004	2.0	0.333	11.5	0.055
<i>K60</i>	2.1	0.004	4.3	0.004	4.1	0.043

Supplementary table 3. Real time qRT-PCR median fold change in the caecums of *C. jejuni* infected birds (n = 7) relative to mock-infected controls at 6 hours, 20 hours and 48 hours post-challenge. *P* values were determined using Mann-Whitney U test.

Innate immune genes	6 hours post-infection		20 hours post-infection		48 hours post-infection	
	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
<i>CD45</i>	2.6	0.025	-1.8	0.180	-1.3	0.180
<i>TLR4</i>	4.0	0.025	1.6	0.180	1.3	0.406
<i>TLR5</i>	3.3	0.180	1.2	0.655	1.2	0.655
<i>TLR15</i>	-1.0	0.749	1.5	0.655	-1.0	0.655
<i>TLR21</i>	3.5	0.025	-1.0	0.655	2.0	0.180
<i>IL1β</i>	-1.8	0.097	5.2	0.005	3.9	0.025
<i>IL6</i>	1.3	0.371	1.3	0.180	4.4	0.025
<i>IFNG</i>	1.5	0.180	1.0	0.798	2.4	0.794
<i>TGFB4</i>	1.7	0.085	-1.3	0.655	1.1	0.201
<i>IL8</i>	-2.9	0.030	24.3	0.002	3.3	0.002
<i>K60</i>	-4.7	0.024	6.6	0.002	1.0	0.749

Supplementary table 4. Real time qRT-PCR median fold change in the caecal tonsils of *C. jejuni* infected birds (n = 7) relative to mock-infected controls at 6 hours, 20 hours and 48 hours post-challenge. *P* values were determined using Mann-Whitney U test.

Innate immune genes	6 hours post-infection		20 hours post-infection		48 hours post-infection	
	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value
<i>IL8</i>	2.3	0.025	4.5	0.002	7.4	0.025
<i>K60</i>	1.7	0.178	1.2	0.653	3.0	0.025