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*Source / Izvornik:* **Virulence**, 2015, 6, 581 - 590

**Journal article, Published version**

**Rad u časopisu, Objavljena verzija rada (izdavačev PDF)**

<https://doi.org/10.1080/21505594.2015.1042642>

*Permanent link / Trajna poveznica:* <https://urn.nsk.hr/urn:nbn:hr:184:909428>

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*Download date / Datum preuzimanja:* **2024-11-23**



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# Virulence genes and cytokine profile in systemic murine *Campylobacter coli* infection

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**Keywords:** *Campylobacter coli*, cytokine profile, mouse model, virulence factors

**Abbreviations:** CDT, cytolethal distending toxin; CFU, colony forming units; IFN, interferon; IL, interleukin; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

*Campylobacter coli* are one of the most common bacteria in bacterial gastroenteritis and acute enterocolitis in humans. However, relatively little is known regarding the mechanisms of pathogenesis and host response to *C. coli* infections. To investigate the influence of genetic changes, we first used PCR to demonstrate the presence of the known virulence genes *cadF*, *virB11*, *cdtB*, *cdtC* and *ceuE* in the clinical isolate *C. coli* 26536, which was isolated from the liver of infected BALB/c mice. Sequence analyses of the *cadF*, *virB11*, *cdtB* and *ceuE* genes in *C. coli* 26536 confirmed the stability in these virulence genes during their transmission through the host. We further investigated *C. coli* infection for the bacterial clearance from the liver and spleen of infected mice, and for their immune response. *C. coli* persisted well in both organs, with better survival in the liver. We also determined the levels of several pro-inflammatory cytokines (i.e., interleukin [IL]-6, IL-12, interferon- $\gamma$ , tumor necrosis factor- $\alpha$ ) and the anti-inflammatory cytokine IL-10 in plasma and in liver homogenates from the infected mice, using enzyme-linked immunosorbent assays. The lowest levels among these cytokines were for tumor necrosis factor- $\alpha$  in the plasma and IL-6 in the liver on days 1, 3 and 8 post-infection. The most pronounced production was for IL-10, in both plasma (days 1 and 8 post-infection) and liver (day 8 post-infection), which suggests that it has a role in healing of the organ inflammation. Our findings showed dynamic relationships between pro- and anti-inflammatory cytokines and thus contribute toward clarification of the healing processes involved in the resolution of *C. coli* infections.

## Introduction

Foodborne illnesses continue to present a public health challenge, and *Campylobacter* spp. represent the most common gastrointestinal bacterial pathogens in the European Union, where infection rates continue to increase.<sup>1</sup> Human infections with *Campylobacter* spp. most often arise as a direct result of consumption of chicken products that have been contaminated during processing<sup>2</sup> and have not been cooked well enough. Intestinal campylobacteriosis manifests as enteritis of the terminal part of the ileum and colon. Patients can have mild to severe symptoms, which commonly include watery, and sometimes bloody, diarrhea, and intense abdominal pain, fever, headache and nausea. Infection is usually self-limiting and lasts only a few days.<sup>3</sup>

Although intestinal infection is rarely associated with a systemic illness, extra-intestinal manifestations or post-infection complications can occur, such as Guillain-Barré syndrome, glomerulonephritis, and reactive arthritis, especially in immunocompromised subjects.<sup>4</sup> Both bacterial and host factors contribute to the differences in the clinical manifestations of such

diseases in human.<sup>5</sup> *Campylobacter* spp. have been reported to have different virulence factors, such as for motility, bacterial adherence to and invasion of epithelial cells, and production of toxins.<sup>5–8</sup> Several pathogenic genes are responsible for these virulence properties, which include: *cadF*,<sup>9</sup> which is responsible for adherence and colony formation; *virB11*,<sup>10</sup> which is responsible for adherence and invasion; *cdtB* and *cdtC*,<sup>11–14</sup> which are responsible for toxin production and have additional roles in adherence and invasion; and *ceuE*, which is responsible for hemolytic activity.<sup>15</sup> However, it is well known that *Campylobacter* spp. are naturally competitive, and combined with their high rate of recombination, this aspect can contribute to their genetic diversity.<sup>16</sup> Although high prevalence of some virulence and toxin genes among *Campylobacter* spp. isolates has been noted, several isolates also show wide variations in the presence of these pathogenic genes.<sup>17,18</sup> The mechanisms that induce this genetic diversity in *Campylobacter* spp. still remain poorly understood, and thus the detection, and a better understanding of, these factors will allow the definition of the virulence factors associated with disease.<sup>18</sup>

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Submitted: 09/11/2014; Revised: 04/02/2015; Accepted: 04/13/2015  
<http://dx.doi.org/10.1080/21505594.2015.1042642>

The interactions of *Campylobacter* spp. with intestinal epithelial cells result in the production of cytokines, which are regulators of host responses to infection, and which have important roles in the pathogenesis of infectious diseases.<sup>19-21</sup> Cytokines are produced by immune cells, and according to their function, they are divided into the pro-inflammatory cytokines, such as interleukin (IL)-1, IL-2, IL-6, IL-8, IL-12, IL-17 and IL-18, tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$ , and the anti-inflammatory cytokines, such as IL-4, IL-10 and tumor growth factor- $\beta$ .<sup>2,22</sup> Both pro-inflammatory and anti-inflammatory cytokines are induced during *Campylobacter* spp infection, and it is important to determine whether the immune system is successful in providing protection against these specific pathogens.<sup>5</sup> Some cytokines can be produced in excess, and thus these might contribute strongly to disease pathology.<sup>19</sup> Indeed, it has been reported that macrophages produce IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  when infected with *Campylobacter* spp.<sup>23</sup> However, the pathogenesis of *C. coli* infection is still poorly understood, which is mainly a consequence of the lack of a suitable animal model that can be used to mimic the course of infection in human.<sup>24</sup>

We thus monitored the stability of the virulence and toxin genes (i.e., *cadF*, *virB11*, *cdtB*, *cdtC*, *ceuE*) by comparing the sequence data of *C. coli* 26536 before and after transmission through the host, and their colonisation and cytokine induction in a murine model. In previous studies, we showed that *C. jejuni* can successfully invade epithelial cells *in vitro*<sup>8,40</sup> and produce systemic infection *in vivo*.<sup>25</sup> Our earlier data obtained *in vitro* showed that *C. jejuni* can survive both in phagocytes (for 72 h) and in intestinal epithelial cells (for 96 h). This is in agreement with the conclusions of Day et al. (2000),<sup>41</sup> who considered *C. jejuni* to be a facultative intracellular pathogen, and not a typical extracellular bacterium.

In the present study, we focused on the clearance from the liver and spleen of BALB/c mice infected with the *C. coli* 26536 clinical isolate, and the association between systemic and local cytokine responses following *C. coli* dissemination. The levels of several pro-inflammatory cytokines (i.e., IL-6, IL-12, TNF- $\alpha$ , IFN- $\gamma$ ) and one anti-inflammatory cytokine (i.e., IL-10) in plasma and liver homogenates were determined on days 1, 3 and 8 post-infection, using enzyme-linked immunosorbent assays. As the cytokine network is one of the main controlling elements in the inflammation process, we wanted to determine any correlation between cytokine production and course of infection.

## Results

### Presence of virulence genes in *C. coli*

The presence of the *cadF*, *virB11*, *cdtB*, *cdtC* and *ceuE* virulence genes in *C. coli* 26536 was analyzed using polymerase chain reaction (PCR) (Table 1). Of these, the *cdtC* gene was not detected in the *C. coli* 26536 strain (Table 2). Furthermore, we analyzed the stability of these genes by sequencing of the virulence genes *cadF*, *virB11*, *cdtB* and *ceuE* in *C. coli* 26536 after transmission through the mice. These examinations of the DNA sequence data of each of these individual genes allowed

comparisons of the analyzed gene sequences in *C. coli* 26536 obtained after 48 h of cultivation on Columbia selective agar in a microaerobic atmosphere at 42°C, and in *C. coli* 26536 isolated from the spleen of infected BALB/c mice. We confirmed the stability of the tested *cadF*, *virB11*, *cdtB* and *ceuE* genes in *C. coli* 26536 (Table 2).

### Survival of *C. coli* 26536 in the livers and spleens of infected mice

BALB/c mice were infected intravenously with *C. coli* 26536 and the course of infection was monitored over 8 days, in terms of the bacterial colony forming units (CFU) in the livers and spleens of the infected mice. Infected mice did not show signs of diarrhea, but they were lethargic with bristled fur, and they appeared not to be in good health (e.g., also showing lack of appetite and shaking). On days 1, 3 and 8 post-infection, the mice were sacrificed and the culturability of the bacteria from their livers and spleens was determined.

On day 3 post-infection, the livers of infected mice were enlarged and yellowish nodes were noted (Fig. 1). Microscopic examination of haematoxylin and eosin stained sections showed local tissue necroses and inflammatory infiltration, with neutrophils and rare plasma cells in the surrounding tissue, although bacteria could not be seen. These macroscopic liver changes were more prominent than those of the spleen. However, *C. coli* 26536 was detected in both livers and spleens of the infected mice through the entire experimental period (Fig. 2), although the growth patterns differed. At each time point, the CFU of *C. coli* 26536 isolated from the spleen was significantly lower than the CFU of *C. coli* 26536 recovered from the liver ( $P < 0.005$ ). *C. coli* 26536 reached their maximal CFU in the liver on day 1 post-infection, which then slowly, but significantly, decreased through day 3 to day 8 post-infection ( $P < 0.05$ ). In contrast,

*C. coli* 26536 CFU in the spleen increased significantly through day 3 to day 8 post-infection ( $P < 0.05$ ), which was the last monitored day of infection in the present study. Kupffer cells in the liver and macrophages, and other phagocytic cells in the spleen, appeared to provide a reservoir where *C. coli* survived for some time, and consequently were isolated from these organs up to day 8 post-infection.

### Levels of different cytokines in the plasma and liver during *C. coli* 26536 infection of mice

The impact of *C. coli* 26536 on systemic cytokine production and in the liver of infected mice was studied, in comparison with the cytokine production patterns in the plasma and liver of non-infected mice. We determined the concentrations of the pro-inflammatory IL-6, IL-12, TNF- $\alpha$  and IFN- $\gamma$ , and the anti-inflammatory IL-10, in the plasma and in liver homogenates on days 1, 3 and 8 post-infection (Fig. 3).

When the mice were infected with *C. coli* 26536, the levels and the dynamics of the production of these investigated cytokines differed. The differences in the concentrations of IL-6 and TNF- $\alpha$  in the plasma between the infected and control mice were minimal throughout the experimental period. TNF- $\alpha$  in

**Table 1.** PCR primer pairs and conditions<sup>32</sup>

Target	Primer pair	n-mer	Sequence (5'–3')	Amplification size (bp)	PCR conditions	Reference
<i>cadF</i>	F2B	20	TGG AGG GTA ATT TAG ATA TG	400	94°C, 1 min	Konkel et al., 1999
	R1B	20	CTA ATA CCT AAA GTT GAA AC		45°C, 1 min (30 cycles) 72°C, 3 min	
<i>virB11</i>	virB11	23	GAA CAG GAA GTG GAA AAA CTA GC	708	95°C, 2 min	Bacon et al., 2000
	virBR	21	TTC CGC ATT GGG CTA TAT G		95°C, 30 s 52°C, 30 s (35 cycles) 72°C, 2 min	
<i>cdtB</i>	VAT2	24	GTT AAA ATC CCC TGC TAT CAA CCA	495	94°C, 1 min	Pickett et al., 1996
	WMI-R	24	GTT GGC ACT TGG AAT TTG CAA GGC		42°C, 2 min (30 cycles) 72°C, 3 min	
<i>cdtC</i>	WMI-F	24	TGG ATG ATA GCA GGG GAT TTT AAC	555	94°C, 1 min	Pickett et al., 1996
	LPF-X	24	GTT GGC ACT TGG AAT TTG CAA GGC		42°C, 2 min (30 cycles) 72°C, 3 min	
<i>ceuE</i>	COL1	24	ATG AAA AAA TAT TTA GTT TTT GCA	894	95°C, 30 s	Gonzales et al., 1997
	COL2	21	ATT TTA TTA TTT GTA GCA GCG		57°C, 30 s (30 cycles) 72°C, 1 min	

the plasma remained at about 22 pg/mL and IL-6 at about 100 pg/mL. On days 1 and 3 post-infection, the levels of IL-12 in the plasma tended to be lower in the infected mice than in the control mice, but by day 8 post-infection, IL-12 in the plasma was higher in the infected mice than in the control mice. However, for the plasma levels of the cytokines, on day 3 post-infection there were also significant differences between the mice infected with *C. coli* 26536 and the control mice, with IFN- $\gamma$  significantly higher in the infected mice (Fig. 3A). At the same time, although the concentrations of IL-10 in the plasma of the infected mice were higher than in the control mice, these differences did not reach significance.

Different cytokine production patterns were revealed in the liver of the mice infected with *C. coli* 26536, as can be seen in Figure 3B. Here, when IL-10 was analyzed, there were marked differences between the infected and control mice through the entire period of infection. The concentrations of IL-10 in the liver showed an increasing trend, and reached the highest values on day 8 post-infection. In addition, there was a pronounced difference between the infected mice and the control mice for TNF- $\alpha$  production in the liver that was already apparent on day 1 of infection. By the last day of infection, day 8, this TNF- $\alpha$  concentration had dropped, and it was slightly lower in the infected mice compared to the control mice, although this difference was

not significant. The differences in IL-6 production in the liver between the infected and control mice were minimal throughout the entire infection period, at around 50 pg/mL to 100 pg/mL in the infected and control mice, and these differences did not reach significance. Furthermore, no significant differences were seen between the infected mice and the control mice for IFN- $\gamma$  in the liver. These results clearly show different cytokine patterns between systemic and local cytokine production during infection, probably linked to the repair of damage to the affected organs.

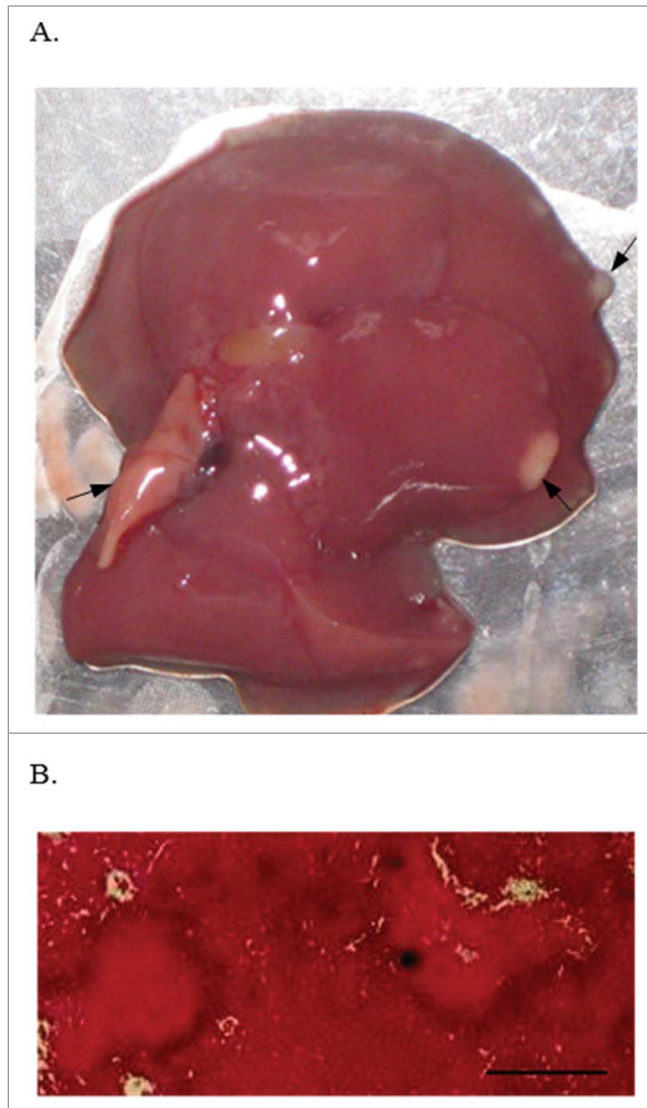
## Discussion

The prevalence of intestinal campylobacteriosis is very high in developed countries, and this represents a great burden for public healthcare systems.<sup>1</sup> Despite this, the pathogenesis of campylobacteriosis is still not completely understood for infections with both *C. jejuni* and, in particular, *C. coli*, although infections with *C. coli* are less frequent than those with *C. jejuni*. Most cases of campylobacteriosis are associated with eating raw or undercooked contaminated poultry or pork meat, or from cross-contamination of other foods by these items.<sup>28</sup> Comparisons of pulsed-field gel electrophoresis and virulence profiles have shown high genetic diversity of the different strains examined, which leads us to

**Table 2.** Target genes and GenBank accession numbers of the *C. coli* 23536 isolate

Virulence properties	Target gene	<i>C. coli</i> 26536		GenBank accession No.
		Isolate from –80°C	Isolate from infected mouse liver	
Adherence, colonisation	<i>cadF</i>	X	X	KJ875966
Adherence, invasion	<i>virB11</i>	X	X	KJ875960
Adherence, invasion, CDT toxin	<i>cdtB</i>	X	X	KJ875957
Adherence, invasion, CDT toxin	<i>cdtC</i>	ND	ND	/
Hemolytic activity	<i>ceuE</i>	X	X	KJ875961

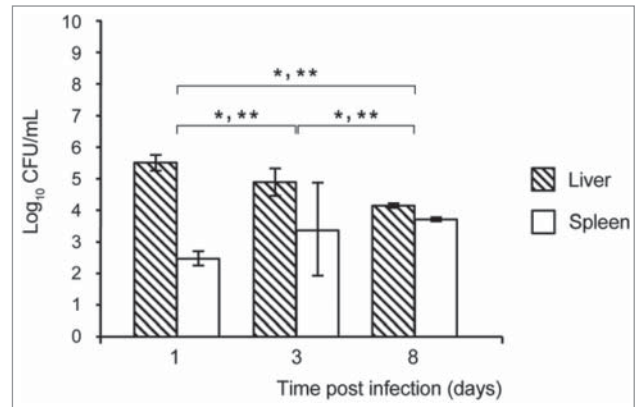
X, gene present; ND, gene not detected.



**Figure 1.** Liver from a BALB/c mouse on day 3 post intravenous infection with  $0.5\text{--}1.0 \times 10^9$  CFU *C. coli* 26536. Macroscopic (A) and microscopic examination of haematoxylin and eosin stained liver section (B). Arrows, local tissue necrosis. Bar, 500  $\mu\text{m}$ .

believe that there can be different degrees of pathogenicity within any given *Campylobacter* spp population.<sup>29</sup>

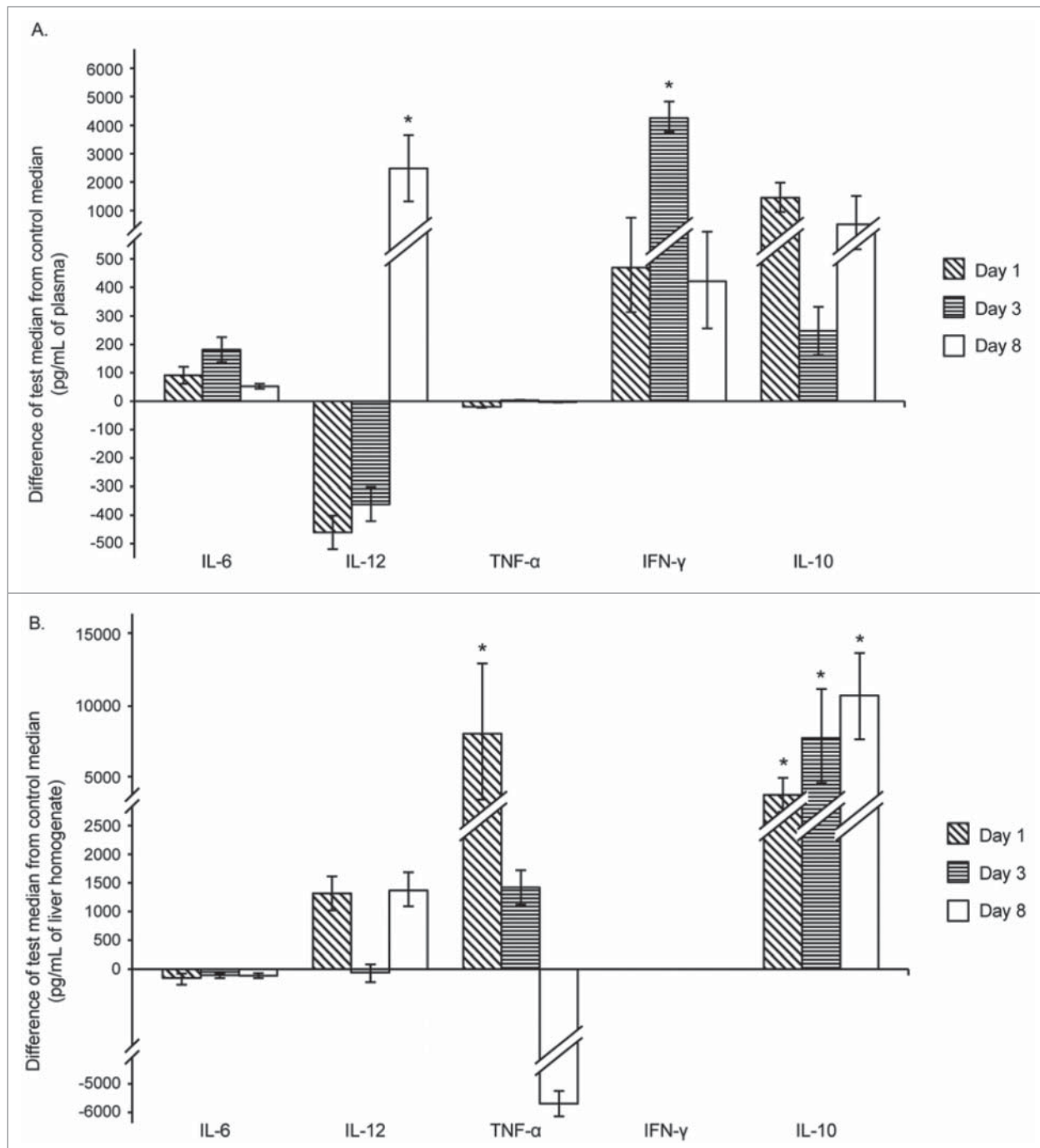
More specifically considering the virulence mechanisms for the pathogenesis of *Campylobacter* spp. during human infections, these might contribute to their flagella-mediated motility, adherence to the intestinal mucosa, invasive capability, and production of toxins.<sup>6,7</sup> Indeed, several potential virulence factors have been identified<sup>11,13-15,23</sup> that show different prevalence according to the human, animal or environmental sources of the *Campylobacter* spp strain.<sup>18,30-32</sup> One of these is the *cadF* gene, which encodes a protein belonging to the group of outer-membrane proteins that facilitates the binding of *Campylobacter* spp. in the host cells,<sup>33</sup> as was confirmed by *Campylobacter* spp colonisation using a chicken model.<sup>34</sup> Another virulence gene linked with



**Figure 2.** Survival of *C. coli* 26536 in the liver and spleen (as indicated) of mice infected intravenously with  $0.5\text{--}1.0 \times 10^9$  CFU *C. coli* 26536. At each indicated time, the mice were sacrificed, and the number of bacteria per organ was determined using the CFU assay. Each point represents the log<sub>10</sub> of the median value  $\pm$  standard deviation, as CFU/organ (\*  $P \leq 0.05$ ).

*Campylobacter* spp. invasiveness is the invasion-associated marker *virB11* gene, which is associated preferentially with both bacterial adherence and invasion.<sup>10</sup> *Campylobacter* spp can also secrete a toxin known as cytolethal distending toxin (CDT), which is a complex of the *cdt* gene products CdtA, CdtB and CdtC. These three subunits act together to block cell division through cell-cycle arrest, and all 3 are necessary for the cytotoxin activity that is known to be lethal for host enterocytes.<sup>11,12</sup> The *ceuE* gene encodes a lipoprotein component of a binding-protein-dependent transport system for the siderophore enterochelin.<sup>35</sup> Iron acquisition is a crucial aspect of bacterial infectivity, and it has been suggested that this system has an important role in bacterial virulence.

In the present study, we initially investigated the presence of the *cadF*, *virB11*, *cdtB*, *cdtC* and *ceuE* genes in the *C. coli* 26536 clinical isolate using PCR. In this way, we confirmed the presence of the *cadF*, *virB11*, *cdtB* and *ceuE* genes, but not of the *cdtC* gene, in this *C. coli* 26536 isolate. Analysis of the *cadF* gene has revealed its high prevalence (i.e., 100%) among *Campylobacter* spp. isolates derived from poultry carcasses, broiler faeces, bovine faeces, retail raw meat, pig and cattle,<sup>18,30-32,36</sup> and also among isolates of human origin.<sup>30,31,37</sup> The *cadF* gene is probably conserved among *Campylobacter* spp isolates,<sup>18</sup> regardless of their origin or species, as is the case also in the *C. coli* 26536 isolate used here. In contrast, several studies have shown wide variations for the presence of *virB11*, which was not found in *C. jejuni* and *C. coli* isolates from poultry faeces,<sup>18</sup> although it was identified in 4% to 15% of isolates tested from poultry meat, pig and cattle,<sup>31,32,36</sup> and in 10% to 17% of human clinical samples.<sup>31,38</sup> However, the prevalence of the plasmid pVir appears to be similar among poultry and human isolates.<sup>17,31,38</sup> The production of the pVir plasmid genes in the *C. coli* 26536 clinical isolate used in the present study thus indicates the possibility of severe invasive infection.



**Figure 3.** Differences from the control mice of the median cytokine levels (as indicated) in the plasma (A) and liver (B) of mice infected with *C. coli* 26536. Median control levels represent zero on the Y axis. Each bar represents the difference between the values for the infected mice minus those for the control mice, at each time point (as indicated) (\*  $P < 0.05$ ). Error bars represent ranges (maximum or minimum test value minus control value). Cytokine levels in control mice were as follows: (A) plasma: IL-6, 12.83 pg/mL; IL-12, 1529.5 pg/mL; TNF- $\alpha$ , 27.61 pg/mL; IFN- $\gamma$ , 42.84 pg/mL; IL-10, 37 pg/mL. (B) liver: IL-6, 853 pg/mL; IL-12, 1659.5 pg/mL; TNF- $\alpha$ , 14445 pg/mL; IFN- $\gamma$ , 3300 pg/mL; IL-10, 1637.8 pg/mL.

In recent studies, the distribution and prevalence of the CDT gene cluster of the *cdtA*, *cdtB* and *cdtC* genes in *C. jejuni* and *C. coli* isolates was investigated, and these genes were found in different ratios.<sup>30,32</sup> The genes encoding the *cdt* gene cluster are widespread among poultry and human *Campylobacter*

spp. isolates, with the *cdtC* gene showing higher prevalence among *C. jejuni* strains (93%–100%) than for *C. coli* (66%–100%), and with similar *cdtB* gene prevalence for both *C. jejuni* and *C. coli* (91%–100%).<sup>18,32,36,37</sup> The absence of the PCR product of the *cdtC* gene in the clinical *C. coli* isolate in the

present study is similar to data from previous studies, where *C. jejuni* can produce much higher levels of CDT in comparison to *C. coli*. The functional toxin is a tripartite complex in which CdtB is the active subunit that has an intrinsic DNase-I-like activity, while the CdtA and CdtC subunits are required for the delivery of CdtB into the host cell.<sup>7,11-13</sup> Furthermore, the interactions of CdtA, CdtB and CdtC are necessary to form an active tripartite holotoxin that has full cellular toxicity.<sup>12</sup> Hence, the absence of the *cdtC* gene indicates that *C. coli* 26536 cannot produce a fully active CDT toxin. The *ceuE* gene encodes a lipoprotein, and this has been detected in all *Campylobacter* spp isolates,<sup>32</sup> and was also present in the clinical *C. coli* 26536 isolate in the present study, thus confirming the high prevalence of this virulence factor.

As determined above, studies of the prevalence of these virulence genes in isolates from various sources are essential to provide more information on the hazards of the *Campylobacter* spp. that might be circulating. As different determinants can influence the characteristics of these virulence determinants,<sup>39</sup> we also followed the stability of the *cadF*, *virB11*, *cdtB* and *ceuE* genes that we detected in *C. coli* 26536 after transmission through a host. Here, we infected BALB/c mice with *C. coli* 26536 and then we collected the isolate from the spleen of the infected mice from day 3 post-infection. Each of the *cadF*, *virB11*, *cdtB* and *ceuE* genes was detected by PCR in isolates after storage conditions at  $-80^{\circ}\text{C}$ , and again in isolates from the livers of these infected mice. Interestingly, all of these virulence genes were maintained in the *C. coli* isolates after the imitation food chain and after the transmission through the host mouse model. We further followed the stability in the *cadF*, *virB11*, *cdtB* and *ceuE* genes by comparing the sequence data for the isolates obtained through the imitation transition route using transmission through the host model. These sequence analyses showed that the sequence data of the virulence and toxin genes *cadF*, *virB11*, *cdtB* and *ceuE* remained the same in both of these isolates, and thus these genes were conserved through the transmission through the host, which supports their involvement in the virulence process.

It is well known that innate immunity is the first line of defense against microbial infection, and that this is required for the removal of the pathogen from the host.<sup>42,43</sup> For colonisation and cytokine induction, we used the experimental model of murine campylobacteriosis, which has been adopted for the study of systemic infection and dissemination of *Campylobacter* spp, and the pathogenesis of *Campylobacter* spp. infection.<sup>25-27</sup> We thus followed the systemic spread and infection for the internal organs (i.e., liver, spleen) after intravenous infection of BALB/c mice with this human clinical isolate of *C. coli*. We followed the course of infection and cytokine production.

After intravenous inoculation, *C. coli* 26536 established both liver and spleen infections, with better survival in the liver than the spleen of these infected mice. The CFU of *C. coli* isolated from the liver of the infected mice on day 8 post-infection was significantly lower in comparison to days 1 and 3 post-infection. This falling pattern of bacterial numbers isolated from the liver has also been seen previously in the liver of mice infected with *C. jejuni*.<sup>44</sup> In contrast, the CFU of *C. coli* isolated from the spleen

of the infected mice in the present study was increasing toward the last day post-infection (day 8). This might be explained by the potentially lower number of macrophages in the liver. Kupffer cells in the liver and macrophages and other cells in the spleen (i.e., neutrophils, eosinophils, monocytes) also have phagocytic functions.<sup>45</sup> It has been shown that any prolongation of bacterial survival in macrophages increases the probability of their transmission within the host organism, and this has further implications in the pathogenesis of campylobacteriosis.<sup>46,47</sup> Thus these cells might represent a reservoir, whereby the *Campylobacter* spp can survive for some time after intravenous inoculation in an experimental animal, which might be why *C. coli* was still isolated from the selected organs at day 8 post-infection.

During a systemic bacterial infection, numerous pro-inflammatory cytokines are produced, and their blood levels are elevated, although this greatly depends on the microbial characteristics and structure.<sup>43</sup> Furthermore, the immune functions of cytokines result from the coordinated action of T cells, macrophages and dendritic cells, and these largely depend on the recruitment of these cells to the sites of infection and inflammation, and to other pathological lesions.<sup>48</sup> Previously, Hu et al. (2006)<sup>49</sup> demonstrated that *C. jejuni* infection can trigger innate inflammatory responses, and induce human dendritic cell maturation and production of pro-inflammatory cytokines, including IL-6, IL-8, IL-12 and TNF- $\alpha$ . In this way, cytokines can induce *Campylobacter*-specific Th1 effector-cell responses, and contribute to the pathogenesis and clinical symptoms of *Campylobacter* spp. infection. However, the molecular mechanisms underlying the dendritic-cell-mediated induction of adaptive immune responses to *C. jejuni* are not known.<sup>50</sup> Interestingly, in the present study, the numbers of bacteria infecting the liver and spleen did not completely correlate with the cytokine profiles in the plasma and liver. Pro-inflammatory cytokines persisted during the whole infection period, both in the plasma and the liver, although *C. coli* 26536 did not provoke the production of IFN- $\gamma$  in the liver, and there was only weak production of IL-12. In addition, high TNF- $\alpha$  production on day 1 post-infection was accompanied by the lowest production of the anti-inflammatory IL-10 in the liver of the infected mice, although not in their plasma.

The spectrum of cytokines in the plasma did not completely correlate with the cytokine profile in the liver. In plasma, for example, the levels of TNF- $\alpha$  were similar to those in the control, non-infected, mice during the whole period of infection, but in the liver, TNF- $\alpha$  peaked on day 1 post-infection and then dropped to even below the level of TNF- $\alpha$  in the control mice. Previous studies have demonstrated central roles for IFN- $\gamma$ , IL-1 and TNF- $\alpha$  in inflammatory reactions, which can lead to the eradication of obligate and facultative intracellular pathogens.<sup>51,52</sup> TNF- $\alpha$  has been shown to have a role in limiting the severity of bacterial infections through various mechanisms, which include the selective killing of cells that harbour bacteria, activation of monocytes and granulocytes, and stimulation of specific immune responses. Particularly in infections with facultative intracellular bacteria, anti-TNF- $\alpha$  antibody treatment promoted massive proliferation of the infecting organisms. These

data suggest that the ability to inhibit TNF- $\alpha$  is advantageous to the pathogen.<sup>53</sup> Unexpectedly, in the present study, *C. coli* did not show this effect: in spite of the extremely low concentrations of TNF- $\alpha$  in the liver of infected mice on day 8 post-infection, the number of *C. coli* isolated also dropped.

Overall, following the infections with *C. coli*, the levels of IL-6 for both the plasma and the liver of the infected mice were almost the same as in the control mice during the entire course of the infection. Infection of the mice with *C. coli* 26536 provoked the greatest production of IFN- $\gamma$  in the plasma on day 3 post-infection. IFN- $\gamma$  is known to enhance major histocompatibility complex class I and II expression on nucleated cells and to stimulate many effector functions of mononuclear phagocytes. In addition, IFN- $\gamma$  induces the expression of many adhesion proteins on the vascular endothelium, to potentiate the function of TNF- $\alpha$  and to promote T-lymphocyte infiltration at sites of infection. For this reason, these high levels of TNF- $\alpha$  in the liver at the beginning of the infection and IFN- $\gamma$  in the plasma might indicate the activation of macrophages and Th1 cells.<sup>5</sup> Our data show time-dependent secretion of IL-12 and an increase in IL-12 production toward day 8 post-infection in the plasma of the infected mice. Secreted IL-12 can enhance CD4<sup>+</sup> T-cell proliferation and IFN- $\gamma$  production, although this was not seen here. In addition, recent studies have shown that IL-12 mediates dendritic cell activation of natural killer cells, and in this way contributes to initial resistance to bacterial pathogens, and also controls reinfection of *C. jejuni* in mice.<sup>54-56</sup> High levels of production of IL-12 initiate a Th1 polarized adaptive immune response.<sup>49</sup>

Interleukin-10 functions as an anti-inflammatory cytokine, and it is known to suppress production of pro-inflammatory cytokines such as IL-12, TNF- $\alpha$  and IFN- $\gamma$ . In addition, its relationship with some pro-inflammatory cytokines is important for the outcome of infection and sepsis.<sup>48</sup> In the present study, the first cytokine that began to rise in the liver of the infected mice was IL-10. *C. coli* 26536 induced the strongest anti-inflammatory IL-10 response in the liver by the last day of infection (day 8). As indicated above, there was an early rise in TNF- $\alpha$  in the liver, which is as expected, as TNF- $\alpha$  is a prerequisite for the production of other pro-inflammatory cytokines. On the other hand, an early increase in IL-10 is hard to explain, especially as it occurred simultaneously with the rise in the pro-inflammatory cytokines, as seen in the liver of the infected mice. This will need further investigation for a systematic explanation. However, this simultaneous production of both pro-inflammatory and anti-inflammatory cytokines has also been demonstrated in an *in-vitro* cell culture model of the INT407 human intestinal cell line.<sup>57</sup>

In conclusion, along with *C. jejuni*, *C. coli* are one of the most common bacteria that cause bacterial gastroenteritis and acute enterocolitis in human in developed countries. Despite its obvious global importance, relatively little is known regarding the mechanisms of pathogenesis and host response to *C. coli* infection. In the present study, we have confirmed the presence of the *cadF*, *virB11*, *cdtB* and *ceuE* virulence genes in this *C. coli* 26536 isolate, and we monitored the stable virulence and toxin genes following *C. coli* transmission through a host, as such differences might influence *Campylobacter* spp colonisation and

cytokine induction in an *in-vivo* model. Furthermore, we used our previously established experimental model of systemic campylobacteriosis in mice. In this way, we followed the course of infection and cytokine production after intravenous challenge of BALB/c mice with this human clinical *C. coli* 26536 isolate. The levels of several pro-inflammatory cytokines (IL-6, IL-12, TNF- $\alpha$ , IFN- $\gamma$ ) and one anti-inflammatory cytokine (IL-10) in plasma and liver homogenate were determined on days 1, 3 and 8 post-infection using enzyme-linked immunosorbent assays. The *C. coli* produced systemic infection in these immunocompetent BALB/c mice, but did not induce production of the pro-inflammatory cytokine IL-6, either in the plasma or in the liver of the infected mice. In contrast, in these infected mice, the anti-inflammatory cytokine IL-10 showed markedly greater production in the liver, with dynamics that correlated with the declining number of *C. coli* in the liver. It is well known that the cytokine network is one of the main controlling elements in the inflammation and immune reactions that occur during bacterial infection. Dynamic relationships between pro- and anti-inflammatory cytokines, their rates of production, and their levels in different tissues are among the variables in the control of healing processes and the resolution of infections. Thus, it is important to determine any correlations between cytokine production and the course of infection. Here, our investigations have contributed further toward the clarification of these relationships.

## Materials and Methods

### Bacterial isolate

The *C. coli* 26536 clinical isolate was provided by the Laboratory for Diagnostics of Enteric Infections of the Teaching Institute of Public Health of the Primorsko-Goranska county, Croatia, and was previously characterized.<sup>58</sup> The isolate was then stored at  $-80^{\circ}\text{C}$  in brain–heart infusion broth supplemented with blood (5%, Oxoid, SR0048) and glycerol (10%, Kemika, 0709704) until use, and cultured using microaerobic growth (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>) in Preston broth (Oxoid, CM0689) at  $42^{\circ}\text{C}$ .<sup>59</sup>

### Virulence genes and sequence analysis

The *cadF* gene encodes adhesin, which is responsible for certain steps in cell invasion, while the *virB11* gene is a marker for the pVir plasmid, the *cdtB* and *cdtC* genes are involved in CDT production, and the *ceuE* gene encodes a lipoprotein that is associated with haemolysins. These five genes were assessed using PCR. For the present analysis using PCR and for the sequence analysis, we used *C. coli* 26536 revitalised on Columbia selective agar (Oxoid, CM0331), after storage at  $-80^{\circ}\text{C}$ . This isolate was further used to infect BALB/c mice, and then recovered from the mouse spleen on day 3 post-infection.

The primer sequences of the virulence and toxin genes, the expected sizes of their products, and the cycling conditions for the determination of the presence of these 5 putative virulence factors *cadF*, *virB11*, *cdtB*, *cdtC* and *ceuE* of *C. coli* are listed in Table 1. Bacterial DNA was extracted using the PrepMan Ultra



sample preparation reagent (Life Technologies, 4318930), following the manufacturer recommendations, and the extracted DNA preparations were stored at  $-20^{\circ}\text{C}$ . PCR amplifications were performed in 50  $\mu\text{L}$  reaction volumes that contained 10 $\times$  RED Taq PCR buffer (Sigma-Aldrich GmbH, B5926), 20 mM dNTP (Life Technologies, N8080007), 300 nM forward primer, 300 nM reverse primer (Table 1), 1 U/ $\mu\text{L}$  RED Taq polymerase (Sigma-Aldrich GmbH, D4309), and 5  $\mu\text{L}$  DNA lysate. The PCR was performed in a thermal cycler PCR system (2400 GeneAmp; Perkin Elmer). The cycling conditions varied according to the specific gene determination (Table 1). The PCR products were electrophoresed on 2% agarose gels. The virulence genes *cdtB*, *virB11*, *cadF* and *ceuE* of *C. coli* were further investigated by sequence analysis (Macrogen), to study the genetic differences of this *C. coli* isolate following the storage conditions, after sub-cultivation in the food model, and after isolation from the infected mice. The sequences of *cdtB*, *cdtC*, *cadF* and *ceuE* have been deposited in GenBank, with their accession numbers given in Table 2.

### *C. coli* 26536 infection in mice

Eight to 12-week-old BALB/c (H-2<sup>d</sup>) mice were obtained from the Central Animal Facility of the Medical Faculty, University of Rijeka, and the experiments were conducted according to the Guidelines of the International Guiding Principles for Biomedical Research Involving Animals (NCR, 2004). The Ethical Committee of the University of Rijeka approved all of the animal experiments described here.

The mice were given standard laboratory rodent food (Mucedola, 4RF21GLP) and water *ad libitum*. They were infected intravenously via the lateral tail vein with a single dose (200  $\mu\text{L}$ ) of  $0.5\text{--}1.0 \times 10^9$  CFU *C. coli* 26536 cells, as determined by the turbidity of the bacterial suspension and confirmed retrospectively by plating the inoculum on blood agar and incubating them microaerobically for 48 h at  $42^{\circ}\text{C}$ . On days 1, 3 and 8 post-infection, the mice were anaesthetised with sodium pentobarbital and sacrificed, and their livers and spleens were removed aseptically. The *C. coli* 26536 CFU in these organs were determined as previously described.<sup>25</sup> At least 6 mice per time point were infected. For organ burden determination, at least 3 mice were sacrificed per time point, and at least another 3 for cytokine analyses. All of the experiments were repeated 3 times and the data from all of the replicate experiments were pooled and are presented as means  $\pm$  standard deviation.

### Cytokine analysis

For the determination of the cytokine concentrations, mice were anaesthetised with sodium pentobarbital and sacrificed, with

their blood collected in sterile vials (Eppendorf AG, 00298–00) that contained ethylenediamine tetra-acetic acid. The tubes were centrifuged, and the plasma was separated and stored at  $-20^{\circ}\text{C}$  until assayed. The livers of these mice were removed aseptically, dissected from the surrounding tissue, and collected on ice in preweighed sterile vials (Eppendorf AG). The weights of the livers were recorded using an electronic balance (PB602-S). The livers were then frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

The livers were homogenized and the organ supernatants collected as previously described.<sup>44</sup> Briefly, immediately before analysis, the livers were thawed on ice and homogenized in ice-cold phosphate-buffered saline (5 mL per 1 g tissue, wet weight) using a hand-held tissue homogenizer. Following homogenization, the liver samples were centrifuged (14,000  $\times$  g, 10 min,  $4^{\circ}\text{C}$ ) to precipitate the cell debris as a pellet. These supernatants were stored at  $-20^{\circ}\text{C}$  until they were assayed for cytokine levels. The levels of IL-6, IL-10, IL-12, TNF- $\alpha$  and IFN- $\gamma$  were determined in duplicate, using mouse cytokine enzyme-linked immunosorbent assay kits (Thermo Scientific, EM2IL6, EM2IL10, EMIL12TOT2, EMTNFA5, ESS0020), and the data are expressed in pg/mL plasma or tissue homogenate, as described previously.<sup>44</sup> The sensitivity levels were: IL-6, 7 pg/mL; IL-10, 12 pg/mL; TNF- $\alpha$ , 9 pg/mL; and IFN- $\gamma$ , 10 pg/mL. Control mice of the same age and sex were injected with sterile saline. All of the experiments were independently repeated 3 times, and the data are presented as means  $\pm$  standard deviation.

### Statistical analysis

The differences for the CFU in the organs and the cytokine levels in the plasma and liver homogenates were calculated using Kruskal Wallis tests. Mann Whitney tests were used to verify differences between pairs of groups. Differences were considered significant at the *P* level of 0.05. Statistical analysis was performed using SPSS 15.0 for Windows (Statsoft Inc.).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Funding

This study was supported by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia, by the Z1–2190 post-doctoral project of AK, by research program P4–0116, and through the research project Molecular Mechanisms of Bacterial Pathogenesis and Stress Response, supported by the University of Rijeka, Croatia.

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