Investigations on the humoral and cell-mediated immune response in chicken after inoculation with *Campylobacter jejuni* strains of human and avian origin

Thesis
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My family
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Publications

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<tr>
<td>AFLP</td>
<td>amplified fragment-length polymorphism</td>
</tr>
<tr>
<td>CCDA</td>
<td>charcoal cefoperazone deoxycholate agar</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>Cdt</td>
<td>cytolethal distending toxin</td>
</tr>
<tr>
<td>CfU</td>
<td>colony forming units</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td><em>Campylobacter jejuni</em></td>
</tr>
<tr>
<td>CKC</td>
<td>chicken kidney cells</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CT</td>
<td>cholera toxin</td>
</tr>
<tr>
<td>CxCL</td>
<td>CXC-motive-chemokine</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>days post inoculation</td>
</tr>
<tr>
<td>E.coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td><em>e.g.</em></td>
<td>exempli gratia</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>x g</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>H₂S</td>
<td>Hydrogen-sulfide</td>
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<tr>
<td>IBDV</td>
<td>Infectious bursal disease virus</td>
</tr>
<tr>
<td>IEL</td>
<td>intraepithelial lymphocytes</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
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<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
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<tr>
<td>IgY</td>
<td>immunoglobulin Y</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LT</td>
<td><em>E. coli</em> heat labile toxin</td>
</tr>
<tr>
<td>LPL</td>
<td>lamina propria leukocytes</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted desorption ionization time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>MLST</td>
<td>multilocus sequence typing</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer membrane protein</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood leukocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>pi</td>
<td>post inoculation</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen-free</td>
</tr>
<tr>
<td>SPRD</td>
<td>spectral red</td>
</tr>
<tr>
<td>ssp.</td>
<td>subspecies</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>THP-1</td>
<td>human acute monocytic leukaemia cell line</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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</tbody>
</table>
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1 Introduction

Today *Campylobacter jejuni* (*Cj.*) is the most common food-borne pathogen being responsible in humans for both acute gastroenteritis as well as long-term consequences such as Guillain-Barré-Syndrome or Miller-Fisher-Syndrome. *Cj.* is found ubiquitous in the environment and in the gastro-intestinal tract of mammals and birds. Birds, and especially poultry is regarded as the main reservoir for this zoonotic pathogen. However, many sources for human *Cj.*- infections are known. The consumption of uncooked or inadequate prepared poultry meat is responsible for most *Cj.* outbreaks in humans.

*Campylobacter* can be found in every poultry production form. Poultry is normally being colonised without induction of clinical signs or pathological lesions. Some authors even regard *Cj.* as a commensal of the intestinal flora of chicken. This complicates the detection and subsequent control of *Cj.* in poultry flocks.

On the one side there is still a lack of knowledge about the factors contributing to colonisation of the avian intestinal tract making abatement even more difficult. On the other hand there is limited information on the local immune response in the chicken gut after *Cj.* challenge.

The origin of the *Campylobacter* strain and the host species as well as host specific factors may play an important role in colonisation, transmission and clinical outcome. There are indications for a strain and host dependent susceptibility and resistance in chicken.

So far most investigations focused on the humoral immune response in chicken after *Cj.* challenge. Due to findings in human patients suffering from campylobacteriosis a significant role of the T cell mediated immunity is also suggested for chicken. Therefore we compare strains of human and avian origin for their colonisation ability in the chicken intestine and furthermore we investigated specifically the role of T helper and cytotoxic T cells in the chicken gut after *Cj.* inoculation.
The results of this study will provide more information on the bacteria-host-interaction and finally give a better understanding of \textit{Cj}. strain-and-host-interaction. We will furthermore shed more light into the controversial discussion about \textit{Cj}. being a commensal or a pathogen for poultry. Finally our results might contribute to the development of control strategies on farm level and food-processing chain to reduce \textit{Campylobacter} contamination of poultry products.

This project was part of a consolidation of members from human and veterinarian medicine. This consolidation included, beside the Clinic for Poultry at the University of Veterinary Medicine, Hannover, working groups from the Institute of Medical Microbiology at the University Medical Centre, Göttingen, the Department of Microbiology and Hygiene at the Charité-Univ.

This network (“CampyGerm”) was funded by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG).
2 Literature review

In poultry, Campylobacter is a widespread and commonly detected bacterium. The major species to be found is Cj. Due to the fact that Campylobacter has a high zoonotic potential and is frequently found on poultry meat, gives raise to investigate Campylobacter not only from the perspective of human medicine but also poultry.

2.1 Campylobacter

2.1.1 Etiology and taxonomy

Formerly being part of the genus Vibrio, taxonomy started to change in 1963 (SEBALD u. VÉRON 1963). During this period the genus covered only two species, namely C. fetus and C. bubulus. Despite the taxonomic changes initiated by Sebald and Véron (SEBALD u. VÉRON 1963) it took another ten years until interest increased in Campylobacter by the work of Butzler in 1972/73, which demonstrated its importance in human bowel diseases (DEKEYSER et al. 1972; BUTZLER et al. 1973). Partial sequencing of 16S rRNA of different Campylobacter species and comparison to previously published sequences of C. laridis, C. jejuni, C. coli and C. pylori identified new relationships and allowed classification at phylogenetic level within the genus Campylobacter (PASTER u. DEWHIRST 1988).

Today the genus Campylobacter spp. includes 25 species and 8 sub-species (MAN 2011). An overview of the known species and sub-species is given in Table 1. Furthermore thermophilic and non-thermophilic species can be differentiated based on their preference for specific growing temperature.

The group of thermophilic Campylobacter consists of four species including C. jejuni, C. coli, C. lari and C. upsaliensis of which C. jejuni is being the most important in poultry (EYERS et al. 1994). Thermophilic species grow at temperatures ≥ 37°C, preferably at 42°C. By contrast the group of non-thermophilic species show growth below 37°C. All species require microaerophilic conditions with 10% carbon dioxide.
(CO₂), 5% oxygen (O₂) and 85% hydrogen for growing (SKIRROW 1977; BOLTON et al. 1984).

Table 1: *Campylobacter* species, their possible hosts and their incidence in humans

<table>
<thead>
<tr>
<th>Thermophilic <em>Campylobacter</em> spp.</th>
<th>Host</th>
<th>Incidence in humans</th>
</tr>
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<tbody>
<tr>
<td><em>C. coli</em></td>
<td>chicken ; turkey ; dog ; duck ; goat ; monkey ; pig ; seagull</td>
<td>√</td>
</tr>
<tr>
<td><em>C. coli</em> hyoilei</td>
<td>sheep ; cattle</td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em> ssp. <em>jejuni</em></td>
<td>chicken ; dog ; duck ; goat ; monkey ; Northern elephant seal ;</td>
<td>√</td>
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<tr>
<td><em>C. jejuni</em> ssp. doylei</td>
<td>sheep ; cattle</td>
<td></td>
</tr>
<tr>
<td><em>C. lari</em> ssp. <em>concheus</em></td>
<td>chicken ; dog ; duck ; foal ; horse ; long-eared owl ; North. elephant seal ; rhesus monkey ; sheep ; wild birds ; mussels ;</td>
<td>√</td>
</tr>
<tr>
<td><em>C. lari</em> ssp. <em>lari</em></td>
<td>water ; cattle</td>
<td></td>
</tr>
<tr>
<td><em>C. upsaliensis</em></td>
<td>cat ; dog</td>
<td>√</td>
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<table>
<thead>
<tr>
<th>Non-thermophilic <em>Campylobacter</em> spp.</th>
<th></th>
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<tbody>
<tr>
<td><em>C. avium</em></td>
<td>chicken ; turkey</td>
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<tr>
<td><em>C. canadensis</em></td>
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<tr>
<td><em>C. concisus</em></td>
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</tr>
<tr>
<td><em>C. cuniculorum</em></td>
<td>rabbit</td>
</tr>
<tr>
<td><em>C. curvus</em></td>
<td>dog</td>
</tr>
<tr>
<td><em>C. fetus</em> ssp. <em>fetus</em></td>
<td>cattle ; horse ; kangaroo ; pet turtle ; sheep</td>
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<td><em>C. fetus</em> ssp. <em>veneralis</em></td>
<td>cattle</td>
</tr>
<tr>
<td><em>C. gracilis</em></td>
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<tr>
<td><em>C. hominis</em></td>
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<td><em>C. helveticus</em></td>
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<tr>
<td><em>C. hyointestinalis</em> ssp. <em>hyointestinalis</em></td>
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<tr>
<td><em>C. hyointestinalis</em> ssp. <em>lawsonii</em></td>
<td>cattle ; dog ; hamster ; moluccan rusa deer ; pig ; reindeer ; sheep</td>
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<tr>
<td><em>C. insulaenigrae</em></td>
<td>North. elephant seal ; porpoise ; South. american sea lion ; wild common seal</td>
</tr>
<tr>
<td><em>C. lanienae</em></td>
<td>cattle ; pig ; sheep</td>
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<tr>
<td><em>C. mucosalis</em></td>
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<td>albatross ( black-browed / grey-headed ) ; gentoo penguin</td>
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<td><em>C. troglodytis</em></td>
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<td><em>C. ureolyticus</em></td>
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</tr>
<tr>
<td><em>C. voelciris</em></td>
<td>Black-headed gull</td>
</tr>
</tbody>
</table>

**provisional species**

| C. sp. *Dolphin*                     | dolphin | --- |
| C. sp. *Prairie Dog*                 | prairie dog | --- |

This table gives an overview of thermophilic and non-thermophilic *Campylobacter* species and subspecies. Shown are the possible hosts of the *Campylobacter* species, and if they may colonise humans. Modified from (MAN 2011). --- = no data available.
Gram staining procedures reveal gram-negative curved rods with a corkscrew- or s-curved-like appearance, a length of 0.2-0.5 μm and a width of 0.2-0.9 μm. *Campylobacter* spp. features a single polar flagellum leading to a corkscrew-like motility. But also more coccoid forms may occur due to age, stress or deleterious impact (SMIBERT 1978; SHANE 1992). Further properties of this non-spore forming bacteria include sensitivity to desiccation, oxygen, heat and low pH (PARK 2002).

### 2.1.2 Colonisation factors

#### 2.1.2.1 In human

The role of cytotoxic distending toxin (Cdt) is discussed to contribute to colonization and virulence in campylobacteriosis of human. *In vitro* studies with human embryo intestinal cells identified Cdt-mediated releases of IL-8 from intestinal cells leading to inflammation (HICKEY et al. 2000). Beside the mediation of cytokine release, Cdt also directly affects live cells. It causes cell distension and cell death (ASAKURA et al. 2007). Intracellular survival of *Cj.* is also apparent in human infections. *Cj.* is phagocyted by human peripheral blood monocytes. After uptake they transform to a coccoid form. At this stage they remain viable and persist (KIEHLBAUCH et al. 1985).

#### 2.1.2.2 In chicken

Many factors have been identified contributing to colonisation in the chicken. Table 2 summarizes factors, which have so far been detected in connection with the avian gastrointestinal tract.
Table 2: *Cj.* Colonisation factors and their related genes expressed in the chicken intestinal tract

<table>
<thead>
<tr>
<th>Function</th>
<th>related gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>flaA</td>
<td>(WASSENAAR et al. 1993; JONES et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>maf5</td>
<td>(JONES et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>flgR</td>
<td>(HENDRIXSON u. DIRITA 2004; WOSTEN et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>flgK</td>
<td>(HENDRIXSON u. DIRITA 2004; FERNANDO et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>cji321-cji325/6</td>
<td>(HOWARD et al. 2009)</td>
</tr>
<tr>
<td>Multidrug efflux pump</td>
<td>cmeABC</td>
<td>(LIN et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>cbrR</td>
<td>(RAPHAEL et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>cmeR</td>
<td>(GUO et al. 2008)</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>docB</td>
<td>(HENDRIXSON u. DIRITA 2004)</td>
</tr>
<tr>
<td></td>
<td>docC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cheY</td>
<td>(QUINONES et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>luxS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cheB</td>
<td>(KANUNGPEAN et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>cheR</td>
<td></td>
</tr>
<tr>
<td>Adhesion</td>
<td>cadF</td>
<td>(FLANAGAN et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>plcA</td>
<td>(ZIPRIN et al. 1999; ZIPRIN et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>peb1A</td>
<td>(DEKKER 2000; ZIPRIN et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>lipA</td>
<td>(LEON-KEMPIS et al. 2006; FLANAGAN et al. 2009)</td>
</tr>
<tr>
<td>Invasion</td>
<td>ciaB</td>
<td>(ZIPRIN et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>docB</td>
<td>(HENDRIXSON u. DIRITA 2004; VEGGE et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>docC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tlp1</td>
<td>(VEGGE et al. 2009; HARTLEY-TASSELL et al. 2010)</td>
</tr>
<tr>
<td>Capsule formation and N-linked glycosylation</td>
<td>kpsm</td>
<td>(JONES et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>pglH</td>
<td>(HENDRIXSON u. DIRITA 2004; KARLYSHEV et al. 2004)</td>
</tr>
<tr>
<td>Two-component regulatory system</td>
<td>racR-racS</td>
<td>(BRAS et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>cbrR</td>
<td>(RAPHAEL et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>flgR-flgS</td>
<td>(HENDRIXSON u. DIRITA 2004; WOSTEN et al. 2004)</td>
</tr>
<tr>
<td>Temperature regulation and heat shock response</td>
<td>dnaJ</td>
<td>(BUTZLER et al. 1973; KONKEL et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>racR</td>
<td>(BRAS et al. 1999)</td>
</tr>
<tr>
<td>Iron regulation</td>
<td>feoB</td>
<td>(NAIKARE et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>fur</td>
<td>(PALYADA et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>cfrB</td>
<td>(XU et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>chuA</td>
<td>(WOODALL et al. 2005)</td>
</tr>
<tr>
<td>Oxidative and nitrosative</td>
<td>docA</td>
<td>(HENDRIXSON u. DIRITA 2004; BINGHAM-RAMOS u. HENDRIXSON 2008)</td>
</tr>
<tr>
<td>Stress response</td>
<td>cji0358</td>
<td>(WOODALL et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>ahpC</td>
<td>(PALYADA et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>katA</td>
<td>(BARNES et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>ggt</td>
<td>(RAJASHEKARA et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>tatC</td>
<td></td>
</tr>
</tbody>
</table>
Central intermediary and energy metabolism

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>frdABC operon</td>
<td>(WEINGARTEN et al. 2009)</td>
</tr>
<tr>
<td>aspA</td>
<td>(WOODALL et al. 2005; GUCCIONE et al. 2008)</td>
</tr>
<tr>
<td>fdhABCD operon</td>
<td>(WEERAKOON et al. 2009)</td>
</tr>
<tr>
<td>nrfA</td>
<td>(WEINGARTEN et al. 2008)</td>
</tr>
<tr>
<td>livJ</td>
<td>(HENDRIXSON u. DIRITA 2004)</td>
</tr>
</tbody>
</table>

Modified from (HERMANS et al. 2011b)

However, none of these colonisation factors seem to be crucial for successful colonisation.

WOSTEN et al. (2004) could show that the flagellum is required in the early stage of colonisation of chicken but is not essential for persistence. Nevertheless, also non-flagellated Cfj. mutants were able to colonise the intestine. This finding indicates that the flagellum is more important for passage than for colonisation (WOSTEN et al. 2004). Motility is strongly connected to chemotaxis, allowing the movement to the lower intestinal tract and so consequently supports colonisation. Mucin was shown to have chemo-attractant properties. It is found in the mucus of the intestinal tract (HUGDAHL et al. 1988).

Cfj. colonisation of chicken usually occurs at the age of two to four weeks (GREGORY et al. 1997; NAUTA et al. 2009; VAN GERWE et al. 2009). Colonisation mainly takes place in the lower intestinal tract, caecum being the predominant organ and reservoir (HERMANS et al. 2011a). Up to $10^8$ colony-forming units (CFU) can be determined in caecal content of broilers (BEERY et al. 1988). Under experimental conditions or due to high infection doses, chickens may be colonised earlier although maternal antibodies are suggested to have a protective effect (CAWTHRAW u. NEWELL 2010).

Infectious doses of approximately 40 CFU are known to be sufficient for a successful establishment of the pathogen in the host under experimental conditions (CONLAN et al. 2007). Knudsen even indicates a minimum infectious dose of 2 CFU for day old chicks (KNUDSEN et al. 2006). Flocks may show an infection rate of up to 100% after Cfj. was introduced (VAN GERWE et al. 2009; ALTER et al. 2011). Additionally colonisation with different strains at the same time may occur (PETERSEN et al. 2001).
2.1.3 Epidemiology

2.1.3.1 Distribution and hosts

*Campylobacter* spp. is found ubiquitous in the environment, and many different species are known to act as reservoirs or are susceptible (Table 1). Wild birds are known to be natural hosts of *Campylobacter*. Among the vast variety of different bird species, members of the family of gulls, cranes, raptors, corvids, waterfowl and passerines are known to harbour *C. jejuni*. Gulls, corvids, raptors and passerines can be found foraging in the surrounding of poultry farms, whereas members of waterfowl and cranes as migratory birds help to disperse the bacteria and may contribute to spread and the introduction to poultry flocks (PACHA et al. 1988; YOGASUNDARAM et al. 1989; CRAVEN et al. 2000; KELLER et al. 2011). *Campylobacter* can be detected in every possible poultry production form.

Within the group of thermophilic *Campylobacter* species, *C. jejuni*, *C. coli* and *C. lari* are found in poultry whereof *C. jejuni* being the most common in both layers and broilers (EYERS et al. 1994). Once *Campylobacter* gets introduced into a flock the prevalence reaches up to 100% (VAN GERWE et al. 2009; ALTER et al. 2011). *C. coli* can be found more often in turkeys than in chicken. Although predominantly appearing in pigs, it can occur in turkeys with a prevalence of almost 50% (HUMPHREY et al. 2007; WEBER et al. 2011). Beside layers, broilers and turkeys other poultry species such as Pekin and Muscovy ducks act as a natural host. Other domesticated animals are known to harbour *Campylobacter* spp. as well such as livestock including sheep, cattle and pig but also cats and dogs.

As already mentioned, pigs are harbouring predominantly *C. coli*, whereas sheep and cattle are regarded as important reservoirs for *C. jejuni* (WEBER et al. 1985a, b). Due to its high prevalence and persistence in the environment and natural animal hosts, control or exclusion of *Campylobacter* from poultry and poultry flocks is very difficult (PETERSEN u. WEDDERKOPP 2001).
2.1.3.2 Transmission and vectors

*Campylobacter* transmission is influenced by multiple factors and a vast variety of sources for infection exists. The circulation and spreading of *Campylobacter* within a poultry flock is still poorly understood. Many possible reasons are suggested, which contribute to transmission and to the entry of *Campylobacter* into a flock (SAHIN et al. 2002).

Beside wild birds, other farm animals may introduce *Campylobacter* to poultry flocks including cats, dogs and other livestock on the farm (SAHIN et al. 2002; GUERIN et al. 2007). Figure 1 shows the interactions and risk factors on the farm level. Transmission occurs horizontally. Birds are mainly colonised asymptomatically most likely via shedding birds and contaminated litter, water or food by oral uptake. From these factors *Campylobacter* contaminated water at drinking sites seems to be one of the major sources for transmission within a flock (MESSENS et al. 2009). Intra-flock transmission between shedding and *Campylobacter*–free birds takes place within days and can lead to an almost 100% positive flock within a week (SHANKER et al. 1990). It is known that *Campylobacter* colonisation is self-limiting. On the other hand, chicken remain colonised till the end of the production cycle, which may be related to re-introduction of the bacteria into the flock or re-infection by shedding birds (GLÜNDER 1994). Persistence in the chicken gut is known for *Cj.* which leads to the problem for keeping poultry flocks completely free from *Campylobacter* (VAN DEUN et al. 2008).

Environmental risk factors for *Cj.* transmission include vehicles, transportation boxes and cages, which can be contaminated due to insufficient hygiene measures (HASTINGS et al. 2011). These may play a role in contamination of *Campylobacter*-free broiler flocks on their way to the abattoir (BULL et al. 2006). Water puddles are also suggested as possible sources for Campylobacter on the farm-level, due to the fact that *Campylobacter* was detected in water puddles before the flocks were tested positive (BULL et al. 2006; MESSENS et al. 2009). Wild mammals (*e.g.* rat, mouse, raccoon) are discussed as important vectors for *Cj.*, but rather for free-range systems. Insects such as house flies, darkling beetles and cockroaches may act as mechanical vectors. Rodents and flies in the vicinity of poultry houses were tested
positive after the flocks had been tested positive for Campylobacter. This suggests that they may not function as a source but more likely act as transmitters (SAHIN et al. 2002; BATES et al. 2004; HALD et al. 2004). A vertical transmission cannot be excluded but seems less important (CALLICOTT et al. 2006).

Figure 1: Risk factors for transmission of Campylobacter on farm level

2.1.3.3 Zoonotic potential

Today Campylobacter is the most commonly notified bacterial zoonotic pathogen in the EU in humans. This increasing trend started in 2005. The reasons for this increase are still indistinct. It is suggested that increased awareness and surveillance led to the rise of reported cases. However, there probably remains a dark figure with undiagnosed or unreported cases (SILVA et al. 2011; EFSA 2012). In 2010 there have been reports of more than 212,000 confirmed cases in the EU. This makes an increase of 6.7% compared to 2009. The notification rate was stated at 48.6 per 100,000 residents for the year 2010 according to reports of 24 member states. This
even resulted in 266 deaths due to *Campylobacter* infection in 2010 (EFSA 2012). In industrialized countries *Campylobacter* as the cause of enteric disease is even more frequent than other bacterial pathogens such as *Salmonella* or entero-pathogenic *E.coli* (ALLOS u. BLASER 1995; WHEELER et al. 1999). There is an ongoing decline of *Salmonella* spp.-infections within the EU, based on strict control programs (EFSA 2012).

The incidence of human *Campylobacter* cases is clearly higher during summer (from June to August). Most cases are reported in children under the age of five. Nevertheless all age groups can be affected (LOUIS et al. 2005; EFSA 2012). Of the reported cases of human campylobacteriosis *C. jejuni* is the most important and most frequently detected species. In 2010 it represented 93.4% of the confirmed cases in the EU (EFSA 2012).

Worldwide, poultry and raw poultry meat are considered to be one of the most important reservoirs for *Cj.* and the most common source for human gastroenteritis (HUMPHREY et al. 2007).

In contrast to poultry, carcasses of pigs and cattle are less *Cj.* contaminated. This might be due to differences in slaughterhouse processing between poultry and other farm animals. The reason for this observation might be faecal contamination during the slaughter process of poultry, which is not common in the slaughter process of cattle and pigs. Further, the drying of the carcasses of cattle and pigs may also play an important role for a lower contamination than in poultry (EFSA 2012). In addition cross-contamination with new strains in the poultry processing plant has been observed. The wet environment and the lack of intensive cleaning and disinfection between the slaughter of different flocks per day might lead to persistence of *Campylobacter* spp. in slaughterhouses (MELERO et al. 2012). While poultry remains positive throughout the whole investigated food-chain, pigs and cattle only showed low numbers of positive samples, mainly during the pre-harvest period. However, there is a great variety in the percentage of *Cj.* positive fresh broiler meat in the member states of the EU ranging from 3.1% to 90% with a mean of 30% (EFSA 2012).
Beside meat products, especially from poultry, other animal products are known to trigger human campylobacteriosis such as raw milk or raw milk products like cheese (BUTZLER u. OOSTEROM 1991; SKIRROW 1991; STANLEY u. JONES 2003; SILVA et al. 2011; EFSA 2012). Eggs do not seem to play a part in Cj.- transmission to humans, however shells can be contaminated by faecal bacteria including Campylobacter spp. (COX et al. 2012). Point of action for the reduction of Campylobacter from food products is therefore high quality in food safety and hygiene (VAN DE GIESSEN et al. 1998; SILVA et al. 2011).

2.1.4 Pathobiology and clinical disease

2.1.4.1. Campylobacter in human

2.1.4.1.1 Pathogenesis

A lot of factors have been shown to contribute to Cj.-mediated illness but none has been proven to be exclusively crucial (ALTEKRUSE et al. 1999). Disease occurs after oral uptake of the pathogen. After entering via the oesophageal route the mucus layer of the distal ileum and colon are colonised at first. Subsequent adhesion to the intestinal cell surfaces takes place leading to functional damage of the epithelial cell layer and disturbance of the normal nutrient absorption. Cj.-invasion or produced toxins as well as subsequent inflammatory processes may contribute to the development of the disease (KETLEY 1997; WOOLDRIDGE u. KETLEY 1997). The microbiota seems to play a crucial role in pathogenicity of Cj. Conventional specific pathogen-free (SPF) mice show colonisation resistance against Cj., whereas gnotobiotic mice enabled colonisation of the same Cj. strain (BERESWILL et al. 2011).

The invasion process has been demonstrated well in vitro using several cell lines including gut tumour cells such as Caco-2 or HeLa cells (EVEREST et al. 1992). In vitro studies have indicated that Cj. may even persist in epithelial cells as a result of avoiding phagocytosis by lysosomes (WATSON u. GALAN 2008).
2.1.4.1.2 Clinical signs

The minimum dose for human infection is stated to be 500 - 800 organisms (BLACK et al. 1988). The incubation period is between 3 to 4 days. The illness is self-limiting with a maximum duration of 6 weeks but usually it is confined to 5 – 8 days (PETERSON 1994; VAN VLIET u. KETLEY 2001). The most frequent clinical manifestation is enterocolitis with symptoms such as watery to bloody diarrhoea, abdominal cramps and pain, fever, anorexia and headache (PETERSON 1994; BLASER 1997). Cj. is also considered as a pre-disposing factor for complications. The most common are neurological diseases like Guillain-Barré- Syndrome and Miller-Fisher-Syndrome or reactive arthritis (HANNU et al. 2002; TAKAHASHI et al. 2005; GODSCHALK et al. 2007).

Immunocompromised people, e.g. HIV positive patients, may suffer from a more severe campylobacteriosis than other patients (MORALES et al. 2011).

2.1.4.1.3 Gross pathology and histopathology

In humans C. jejuni may induce severe enteritis of jejunum, ileum and colon with infiltration of inflammatory cells such as neutrophils, eosinophils and mononuclear cells leading to the formation of crypt abscesses (WASSENAAR u. BLASER 1999; MURPHY et al. 2011).

2.1.4.2 Campylobacter in chicken

Among avian species, the chicken considered as the major reservoir for Campylobacter is the most investigated one.

2.1.4.2.1 Pathogenesis

As in humans the mechanisms of pathogenesis remain unclear.

After oral uptake the main localisations of Campylobacter are the deep caecal crypts where they can be found in the mucus near the epithelium (LEE u. NEWELL 2006).
In vitro studies revealed that C. jejuni invades and evades crypt epithelial cells of chicken without causing inflammation, necrosis or apoptosis. Inside the cells they seem not to proliferate. The replication takes place after evasion in the intestinal mucus (VAN DEUN et al. 2008). This seems to be a protective mechanism of Cj. in chicken to avoid clearance, which consequently allows Cj. persistence.

2.1.4.2.2 Clinical signs

Cj. normally colonises chicken without induction of clinical symptoms. In healthy chicken Cj. is regarded as a commensal of the intestinal tract (LEE u. NEWELL 2006). Cj. infection in poultry is mostly self-limiting with a low mortality (GLÜNDER u. WEBER 2000). Formally known as C. fetus ssp. jejuni, Cj. was associated with avian hepatitis (SMITH u. MULDOON 1974; GLÜNDER 1989). Also recent studies still suggest a connection between hepatitis and Cj., but this could never be confirmed directly or being reproduced experimentally (BURCH 2005; JENNINGS et al. 2011).

In several experimental studies clinical signs such as diarrhoea and apathy were induced by Cj. Age has to be regarded as a factor contributing to the development of clinical signs in the host. RUIZ-PALAICOS (1981) induced gastroenteritis in 3-day old chicken using an oral dosage of 9x10^7 bacteria / animal (RUIZ-PALACIOS et al. 1981). In contrast, WELKOS (1984) was not able to induce clinical signs in 3-day old chicks. On the other hand he succeeded to cause diarrhoea in hatchlings at 12 hours post hatch by oral inoculation. In his study he could also show that clinical manifestation seems to be dose related. Higher doses (10^7 and 10^9) led to higher percentage of chicken showing enteritis and an earlier onset of disease than doses of 10^1 to 10^5 CfU (WELKOS 1984).

Beside dosage and age of the chicken, breed also seems to have an impact on the outcome of infection. Two to three day old White Leghorn chicken were less susceptible for Cj.-induced diarrhoea than birds from the Starbro strain. No diarrhoea was induced in White Leghorn by neither of the different Cj. strains inoculated (SANYAL et al. 1984). This observation was supported by a study in which two different broiler lines were Cj. inoculated, which resulted in one line being more
resistant against colonisation than the other (LI et al. 2011). The influence of the host’s genetic background on disease resistance is also known from other avian pathogens such as infectious bursal disease virus (IBDV) (ARICIBASI et al. 2010). Another factor which might influence disease development is the immune status of the host. It was shown that a co-infection with IBDV leads to a higher rate of Cj.-colonisation in birds than mono-infection with Cj. (SUBLER et al. 2006).

2.1.4.2.3 Gross pathology and histopathology

In chicken, Cj.-infection can lead to a mild enteritis of jejunum, ileum or the large intestine with lesions such as blood in the lumen or petechial hemorrhages (RUIZ-PALACIOS et al. 1981; WELKOS 1984). Findings from a slaughter house in Canada suggested that Campylobacter may lead to liver necrosis (BOUKRAA et al. 1991; JENNINGS et al. 2011). However, mono-infection with Cj. does not induce clear clinical signs, which is supported by histological investigations. In single cases of inoculated chicken a mild oedema in the lamina propria of the proximal caecum was detected (BEERY et al. 1988).

2.1.5 Prevention and prophylactic strategies

Controlling Campylobacter transmission and entry into the food-chain is the most important goal. There are several measures to reduce Campylobacter in the processing chain from farm to fork but none is 100% effective.

2.1.5.1 Hygiene and disinfection

High bio-security is the most effective measure to control and reduce Campylobacter in poultry farms (PERKO-MAKELA et al. 2009). Nevertheless, high hygiene standards including disinfecting footbath and working cloth changes between flocks have to be maintained and strictly followed. Normally these on farm measures only lead to a reduction in numbers of Cj.-positive flocks but
not to complete prevention of \textit{Cj.} introduction (VAN DE GIESSEN et al. 1998). The use of chlorinated water at a concentration of 40 mg/ litre at all stages of the slaughter cycle led to a reduction of \textit{Cj.} but it could not fully prevent \textit{Campylobacter} introduction to the processing chain. Cross-contamination between intestinal content and carcass as well as the use of water during processing remains a large issue in prevention and control (MEAD et al. 1995). \textit{Cj.} may even survive the cleaning and disinfection process in poultry slaughterhouses (PEYRAT et al. 2008) which then leads to contamination of \textit{Campylobacter}-free flocks processed afterwards. It is known that \textit{Campylobacter} species are sensitive to low pH, heat and desiccation (PARK 2002). However, disinfection with preservatives with a low pH only showed little success in reducing \textit{Campylobacter}. Acetic acid at a concentration of 1\% was used at different temperatures (4°C, 25°C and 42°C) in both, minced chicken meat and in broth inoculated with \textit{Cj.} While a reduction from $10^8$ down to $10^1$ CfU/ml was observed in broth at 42°C within 24 hours, no effect was seen at temperatures of 4°C and 25°C. This observation was not confirmed after treatment of \textit{Cj.} inoculated chicken meat with 1\% acetic acids. The authors also state, that higher concentrations of acetic acid would allow more efficient killing but would also harm the birds when used at farm level (SHIN et al. 2001).

2.1.5.2 Vaccines

By now there is no commercial vaccine against \textit{Campylobacter} available for chicken. Many approaches have been taken to develop a vaccine against this pathogen. DE ZOETE \textit{et al.} (2007) reviewed the attempts of vaccine development (DE ZOETE et al. 2007). An overview is given in Table 3.
<table>
<thead>
<tr>
<th>Type of vaccine</th>
<th>Administration</th>
<th>Booster</th>
<th>Effect on Campylobacter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune complex vaccine</td>
<td>orally</td>
<td></td>
<td>log 1 reduction</td>
<td>(CAWTHRAW et al. 1998)</td>
</tr>
<tr>
<td>non-colonising mutants of Cj</td>
<td>orally</td>
<td></td>
<td>n.e.</td>
<td>(ZIPRIN et al. 2002)</td>
</tr>
<tr>
<td>Formalin inactivated Cj ; +/- E.coli heat labil toxin</td>
<td>orally</td>
<td>yes</td>
<td>log 1.5 reduction</td>
<td>(RICE et al. 1997)</td>
</tr>
<tr>
<td>Formalin inactivated Cj ; +/- LT or cholera toxin (CT)</td>
<td>orally</td>
<td>yes</td>
<td>n.e.</td>
<td>(CAWTHRAW et al. 1998)</td>
</tr>
<tr>
<td>Formalin inactivated Cj ; + completes Freund’s adjuvant</td>
<td>subcutaneous</td>
<td>yes</td>
<td>reduction of shedding in first 2 weeks, but not of colonisation</td>
<td>(GLÜNDER et al. 1998)</td>
</tr>
<tr>
<td>Heat-killed Cj</td>
<td>in ovo</td>
<td>yes</td>
<td>protection unclear ; induction of IgM, IgA, IgG</td>
<td>(NOOR et al. 1995)</td>
</tr>
<tr>
<td>native flagellin protein ; with and without heat-killed Cj</td>
<td>intraperitoneal</td>
<td>yes</td>
<td>~log 2 reduction</td>
<td>(WIDDERS et al. 1996; WIDDERS et al. 1998)</td>
</tr>
<tr>
<td>recombinant flagellin fused to LT</td>
<td>orally</td>
<td>yes</td>
<td>reduction of Cj positiv chicken</td>
<td>(KHOURY u. MEINERSMANN 1995)</td>
</tr>
<tr>
<td>plasmid DNA vaccine with Cj 11168 flaA gene</td>
<td>intramuscular</td>
<td>yes</td>
<td>log 2 reduction</td>
<td>(NEWELL u. CAWTHRAW 2006)</td>
</tr>
<tr>
<td>immunogenic Cj proteins ( 67, 73.5 , 77.5 kDa )</td>
<td>intraperitoneal</td>
<td></td>
<td>n.e.</td>
<td>(WIDDERS et al. 1998)</td>
</tr>
<tr>
<td>attenuated Salmonella strain expressing C. coli CjA</td>
<td>orally</td>
<td></td>
<td>&gt;log 6 reduction</td>
<td>(WYSZYNSKA et al. 2004)</td>
</tr>
<tr>
<td>CjA-transfected Eimeria tenella</td>
<td>orally</td>
<td></td>
<td>log 1 reduction</td>
<td>(CLARK et al. 2012)</td>
</tr>
</tbody>
</table>

Modified from (DE ZOETE et al. 2007). n.e. = no effect, + = with ; - = without, LT = heat labil toxin, CT = cholera toxin
In some cases a reduction of *Campylobacter* was attained, but no complete protection was accomplished.

In former studies the effect of antibodies on the rate of *Campylobacter* colonisation was demonstrated (CAWTHRAW et al. 1998). Nevertheless, the humoral immune response seems to be not strong enough to prevent colonisation (DE ZOETE et al. 2007).

In several studies the potency of killed whole-cell vaccines (formalin inactivated *Cj.*) was investigated but did not lead to a successful protection against *Cj.* in broilers. There was no protection neither against homologous nor heterologous *Cj.* strains. Only in one study a minor reduction of 1.5 log units of *Cj.* was observed in vaccinated broilers. This poor immunogenicity of inactivated *Cj.* vaccines may be explained by the loss of surface structures by culture derived *Campylobacter*. It is suggested that in consequence of culturing, the strains do not develop surface structures as they might do in the host environment (DE ZOETE et al. 2007).

The flagellin protein is suggested to play an important role for successful *Campylobacter* colonisation (HENDRIXSON u. DIRITA 2004) and it may be a promising vaccine antigen candidate (CAWTHRAW et al. 1994). However, there is a lack of cross-protection due to the high variability of pseudaminic acid residues between different *Cj.* strains (LOGAN et al. 2002; DE ZOETE et al. 2007).

The use of a recombinant attenuated *Salmonella* vaccine expressing the *C. jejuni-cjA* gene induced significant protection against a wildtype *Cj.* strain. Both, a reduction of *Cj.* of approximately 6log10 CfU in broilers and the induction of serum IgG and mucosal IgA antibodies were observed (WYSZYNSKA et al. 2004).

2.1.5.3 Alternative methods

Beside vaccines further approaches are under investigation to reduce *Campylobacter* in poultry.
2.1.5.3.1 Bacteriophages

Bacteriophages are viruses showing the ability to infect and kill susceptible bacterial agents. They can be found ubiquitous in the environment. Due to their properties, they may be interesting candidates for therapeutic use in bacterial infections. Advantages are their high specificity for certain bacterial species and same environmental requirements as their target bacterium. Consequently no side effects are likely to appear, such as damage of the natural intestinal flora. Furthermore they are self-maintaining. They replicate in their host and are only present as long as the targeting bacteria are present (CONNERTON et al. 2011). Phage-therapy of young birds only delayed Cj. colonisation but did not lead to full protection. Treatment of already Campylobacter-positive birds led to a reduction of Cj. in caecal content within 48 hours (WAGENAAR et al. 2005). However, a complete clearance was not observed (WAGENAAR et al. 2005) possibly due to both, developing phage-resistance or loss of phage-susceptibility of Campylobacter (FISCHER et al. 2013).

2.1.5.3.2 Competitive exclusion

Competitive exclusion (CE) is based on the introduction of a protective intestinal flora to prevent colonisation by enteric pathogens such as Campylobacter (MEAD 2000). In one study, the number of Cj. positive birds and caecal colonisation was reduced by the use of a competitive exclusion flora taken from SPF chicken colonised with a protective microflora against Salmonella (SOERJADI et al. 1982). However a CE flora may be passed by using challenge doses of 10⁶ to 10⁸ CFU of Cj. (SOERJADI-LIEM et al. 1984). Other studies showed controversial results possibly due to loss of the protective effect during cold storage of the CE flora (STERN 1994). However, for Klebsiella pneumonia, Citrobacter diversus and E.coli an inhibitory effect against Cj. was observed in a study using one-day-old White Leghorn cockerel chicks. By using a mixture of the three bacteria a protection of up to 100% was obtained. The three mentioned bacteria all utilize mucin as an energy source and therefore metabolize Cj. antagonists. Further they occupy the same niche as Cj.
However, the authors state that further research has to be conducted before the protection induced by these bacteria is fully assessed (SCHOENI u. DOYLE 1992).

2.1.5.3.3 Probiotics

Probiotic bacteria are known to have an influence on pathogenic bacteria. They are able to interact with and to reduce adhesion of virulent bacteria in the intestinal tract. Nowadays they are widely used in human alimentation. The mechanisms involved are competition for binding-sites and sustention sources. Furthermore antimicrobial substances are released, and immune stimulation is initiated by probiotic bacteria, which contributes to the elimination of bacteria (FOOKS et al. 1999).

In chicken beneficial effects of probiotics were shown for several pathogens including other enteric pathogens such as Salmonella (VAN COILLIE et al. 2007).

In several studies Lactobacillus species were identified to have an antagonistic impact on Campylobacter (STERN et al. 2006; MESSAOUDI et al. 2011; GHAREEB et al. 2012).

2.1.5.3.4 Prebiotics

Different feed-additives have been investigated for their protective effect against Cj. in chicken. Carbohydrates such as mannose, lactose and fructooligosaccharides were investigated alone and in combination with CE in one-day old White Leghorn chicks challenged with a Cj. strain. The results showed a significant protection against Cj. in both, the prebiotic single treatment group and in the group which received a combination of prebiotic and CE. Thus, beside the direct protective effect of the investigated carbohydrates against Cj., all three carbohydrates also enhanced the protective effect of the CE. Inhibiting adherence, decreasing caecal pH or the influence on the intestinal flora are suggested as possible anti-Cj.-mechanisms (SCHOENI u. WONG 1994).

A study using glucan and mannan in Cj.-challenged Canaries (Serinus canaria forma domestica) revealed no significant protection against Cj. (AUERBACH u. GLÜNDER 2004).
2.1.6 Diagnostics and detection methods

2.1.6.1 Culture-based isolation

Thermophilic *Campylobacter* spp. requires both, a specific atmosphere (10% carbon dioxide, 5% oxygen and 85% hydrogen) and temperatures between 37°C and 42°C for growing.

Being part of the intestinal flora and mostly isolated from faecal samples, *Campylobacter* also require selective media to eliminate background flora. The first selective medium was developed by Skirrow in 1977. To date approximately 40 different, both solid and liquid media are known for *Cj*. isolation and propagation (Corry et al. 1995). The most commonly used agars are modified charcoal cefoperazone deoxycholate agar (mCCDA), Karmali, Skirrow and Preston. These media contain a selection of antibiotics. *Campylobacter* is for example resistant against nystatine, colistin, cefoperazone or vancomycin (Lai-King et al. 1985; Lai-King et al. 1988).

Growing colonies can differ in their appearance depending on the used media. On mCCDA for example they display flat grey to silvery round smudgy colonies (Bolton et al. 1984).

Fresh cloacal swabs or intestinal content can be plated directly on solid media without previous enrichment. Due to its sensitivity to heat and dryness this method is used to avoid desiccation during transportation of the samples. After being spread on the agar, the plates have to be placed directly in containers with the right atmosphere. Enrichment in liquid media such as Preston broth is used to detect lower numbers of *Campylobacter* in processed food or water. The use of enrichment is discussed controversially. Two studies even suggest that previous enrichment may reduce the detection rate in samples of intestinal content of chicken. In some studies direct plating resulted in better detection rates of *Cj.* in caecal samples than after enrichment (Musgrove et al. 2001; Acke et al. 2009).

Especially for epidemiological investigations it has to be considered that *Campylobacter* spp. may show a viable but non-culturable stage. At this stage it
alters to a more coccoid form. Its favourable environment for the induction of this stage is cold (4°C) and slow moving water due to low oxygen concentrations. By rising the temperature the bacteria may become metabolically active again (ROLLINS u. COLWELL 1986).

2.1.6.2 Use of antibodies for detection / differentiation of *Campylobacter*

Serological tests are used for the diagnosis of disease outbreaks. However, there is a lack of cross-reactivity between *Campylobacter* strains in some cases (ON 1996). For a rapid detection of thermophilic *Campylobacter* species, serological tests have been developed. They are based on the agglutination of cellular antigens of *Campylobacter* species with latex particles coated with immunoglobulins. These immunoglobulins are directed either against several *Campylobacter* species, against numerous *Cj.* serotypes or flagellar antigens (ON 1996). There are also specific antibodies available to identify *Campylobacter* at species-level. These antibodies have to be directed against the outer membrane proteins (OMP's) of *Cj.*, *C.coli* and *C. lari*. These antibodies can be used in enzyme-linked immunosorbent assays (ELISA) or immunoblot (TAYLOR u. CHANG 1987).

2.1.6.3 Biochemical procedures

To identify the *Campylobacter* genus or species level their biochemical activity can be determined. This includes tests for catalase and oxidase activity. Catalase positive species are *C. fetus* ssp. *fetus* and ssp. *veneralis*, *C.jejuni* and *C. coli*. To distinguish these species further, more biochemical properties can be tested by the use of commercially available test kits (Api® Campy, BioMérieux). But the API-Campy system is discussed controversially. In one study the API-Campy system failed to identify more than 50% of the investigated strains. It was shown that matrix-assisted desorption ionization time-of-flight mass spectrometry (MALDI-TOF) is the method of choice for *Campylobacter* species identification, showing an identification rate of 100%. However, it cannot be used to determine antimicrobial resistance of these strains (MARTINY et al. 2011).
In comparison to other Campylobacter species like C. coli or C. fetus Cj. hydrolysis hippurate which can be used for further differentiation (HARVEY 1980; LEAPER u. OWEN 1981). However, other studies revealed aberrations depending on the used hippurate hydrolysis method (MORRIS et al. 1985). Cj. and C. coli both show the ability for H₂S production in contrast to C. fetus (LEAPER u. OWEN 1981).

Antibiotic resistance and susceptibility patterns can be used for further characterization of Campylobacter species. As an example, Cj. and C. coli show resistance to cefoperazon and cephalotin, but sensitivity to nalidixic acid. Other species like C. fetus or C. lari show a contrary resistant pattern (ON 1996). But the rate of nalidixic acid resistant strains increased with nearly 40% for Cj. and between 30 and 50% for C. coli isolates being resistant today. Overall, there is the necessity to combine many biochemical tests for differentiation between different Campylobacter species (REINA et al. 1995).

2.1.6.4 Molecular methods

2.1.6.4.1 Genomics

Sequencing the transcriptome of Campylobacter species offers a new possibility for better understanding of the bacteria. Whole Transcriptome Shotgun Sequencing (RNA-Seq) or DNA-microarray can be used. These techniques even give the possibility to detect and to quantify RNA expressed at very low levels and finally identify differentially expressed genes (CHAUDHURI et al. 2011). High-resolution transcriptome mapping revealed strain-specific transcription patterns. This will help to understand and to elucidate genes which promote phenotypic differences between strains. Up to now still little is known about the transcriptome structure of Campylobacter (DUGAR et al. 2013).
2.1.6.4.2 Proteomics

The investigation of the proteome is based on biochemical analysis of proteins. Techniques for research encompass on the one hand Western Blot or matrix-assisted desorption ionization time-of-flight mass spectrometry (MALDI-TOF) for protein identification. On the other hand methods like Gelelectrophoresis (2D-PAGE) are used for characterization of cellular and membrane proteins. One study investigated the complete protein expression profile of the well-known *C. jejuni* reference strain 11168. This will help to further investigate the pathogenicity of *C. jejuni* (ZHANG et al. 2013).

2.1.6.4.3 Genotyping

Due to the fact that *Campylobacter* spp. are unstable and show a high genetic diversity techniques like multilocus sequence typing (MLST) are becoming more significant (RIDLEY et al. 2008). MLST confirmed the genetic diversity and a high rate of intraspecies recombination of *Campylobacter* (DINGLE et al. 2001; SUERBAUM et al. 2001). Besides MLST, methods like amplified fragment length polymorphism (AFLP) or pulsed-field gel electrophoresis (PFGE) may allow the identification of genetic diversity of *Campylobacter* in poultry flocks (ALTER et al. 2011; PENDLETON et al. 2013). A limiting factor for the application of these methods in the field or in diagnostics remains their high costs per sample.

All these methods may help to determine phylogenetic relationships between campylobacter strains, which will lead to further insights in the origin and possible transmission pathways of *Campylobacter* and finally may allow better control measures (ZAUTNER et al. 2012).
2.2 Immune response

Due to the fact that *Cj.* often leads to a severe illness in human, research on the immune mechanisms, in disease development and recovery are important. On the other side, the investigation of the chicken immune response after *Cj.* inoculation may help to bring up measures for *Cj.* control on the farm level and this way to reduce the incidence in human.

2.2.1 Immune response in human

2.2.1.1 Innate immune response

*Campylobacter* interacts with the intestinal epithelial cells which leads to secretion of pro-inflammatory interleukin (IL) -8 or now better named as CxCL8. Dendritic cells (DC), macrophages and neutrophils are attracted to the side of infection, followed by the release of further pro-inflammatory cytokines such as IL-6, IL-1β, IL-12 and IL-23. An overview of the events is given in figure 2. These released pro-inflammatory cytokines may adjust a T cell mediated immune response (EDWARDS et al. 2010).

The contributing role of monocytes and macrophages to the control of *Campylobacter* remains elusive. They may contribute to inflammation but may also act as a reservoir for *Campylobacter* due to intracellular survival of *Campylobacter* (KIEHLBAUCH et al. 1985; YOUNG et al. 2007).
In a case study it was shown that IL-8 levels peaked at day three after *Cj*- infection of a 46-year-old man. IL-6 and IL-10 as regulatory cytokines were absent at the onset of disease but rose to high levels at day 10 (BAQAR et al. 2001). Edwards (2010) also discusses the role of IFN-γ, IL-22 and IL-17A in *Cj*-infection. He suggests that this triplet may trigger the host anti-microbial immunity. IL-17A may...
also reduce the numbers of intracellular Cj, highlighting the importance of this cytokine in the immune response directed against Cj (EDWARDS et al. 2010). 

Same results could be affirmed in in vitro studies using different human cell lines. Induction of CxCL8 was confirmed for both, Cj-infected human dendritic cell lines and the human monocytic cell line THP-1 (JONES et al. 2003; HU et al. 2012). Beside CxCL8 also IL-1α, IL-1β, IL-6 and Tumour-necrosis factor (TNF) –α were detected in THP-1 cells after infection with different Campylobacter strains. One strain showed aberrant results causing reduced levels of all cytokines indicating strain variability in inducing a host immune response (JONES et al. 2003).

In gnotobiotic mice infected with Cj at the age of 10-12 weeks both, an increase of apoptosis, neutrophils as well as an increase of IL-6, TNF-α and monocyte chemoattractant protein (MCP) -1 was observed in the colon (BERESWILL et al. 2011).

2.2.1.2 Humoral immune response

All types of antibodies (IgM, IgA, IgG) can be detected during a campylobacteriosis. The peak of antibody production was determined at 10 days post-infection. However, there are Campylobacter-strain dependent variations with regard to onset time and antibody titre. Some strains may not induce certain isotypes like IgA. Antibodies are detectable for at least 120 days post infection, however showing a steady decrease (BAQAR et al. 2001).

2.2.1.3 Cell-mediated immune response

T cells are suggested to play an important role in the control of human campylobacteriosis. It is known that HIV patients show more severe disease than people with a normal immune status. Additionally, immunocompromised people are not able to clear the bacterial burden (MORALES et al. 2011).

The uptake of Campylobacter by macrophages, neutrophils and dendritic cells and their cytokine secretion triggers a Th-1 response (YOUNG et al. 2007; EDWARDS et al. 2010).
An increase of T-lymphocytes can be observed in the intestinal tissue after infection with *Cj*. The investigation of rectal biopsies of human patients revealed a duplication of lamina propria T cells (CD4+, CD3+, CD8+). Further, a fivefold increase of cytotoxic (CD8+) T cells was observed in the population of intraepithelial lymphocytes (IEL) (SPILLER et al. 2000). An increase of T and B cells was observed in the colon of mice after infection with a *Cj* strain (BERESWILL et al. 2011). Stimulation of peripheral blood mononuclear cells (PBMC) *in vitro* with *Cj* led to proliferation of TCRγδ T cells also indicating a role of the T cell mediated immune response in *Cj* infection (VAN RHIJN et al. 2003).

### 2.2.2 Immune response in chicken

#### 2.2.2.1 Innate immune response after *Cj* infection in vivo

Four-week old chicken showed after *Cj* challenge with a dose of \( \sim 10^8 \) CfU an increase in pro-inflammatory cytokines beginning at 20 hours post challenge (SHAUGHNESSY et al. 2009). A pro-inflammatory cytokine response was also seen in birds, which had been challenged with *Cj* at day one or at two weeks post hatch (SMITH et al. 2008).

The role of heterophils in *Cj* infection remains unclear. No increase or influx of heterophils was observed after the *Cj* inoculation of four-week-old chicken (HENDRIXSON u. DIRITA 2004; SHAUGHNESSY et al. 2009). This was also confirmed by SMITH *et al.* (2008) in one-day old chicks. Interestingly he detected an increase in heterophils in the caecum of chickens inoculated at the age of two weeks (SMITH et al. 2008).

#### 2.2.2.2 Innate immune response after *Cj* infection in vitro

*In vitro* studies in different avian cell lines such as HD11 macrophages, primary chicken kidney cells (CKC) and primary chicken embryo intestinal cells also demonstrated the induction of an innate immune response after *Cj* infection.
In all these studies a multiplicity of infection of 100 was used to determine the cytokine response. All studies clearly showed an increase of pro-inflammatory cytokines such as IL-6 and IL-1β after \textit{Cj}. infection while no induction of IFN-γ was measured (SMITH et al. 2005; LI et al. 2008).

2.2.2.3 Humoral immune response

Maternal antibodies are suggested to influence the onset of \textit{Cj}. infection. Three-day- and 21-day old broilers showed a different pattern of shedding after inoculation with two different \textit{Cj}. strains at a dose of approximately $10^2$ CFU of \textit{Cj}. The group of three-day-old chicken inoculated with the first strain showed shedding from day seven post inoculation. In contrast the three-day-old birds inoculated with the other strain showed no shedding throughout the experiment. On the other hand both 21-day-old groups shed \textit{Cj}. from day three pi. These findings indicate both, a strain dependent colonisation pattern as well as older birds being more susceptible for \textit{Cj}. infection rather than younger birds (SAHIN et al. 2003). In contrast CAWTHRAW & NEWELL (2010) demonstrated that newly hatched chicks were as susceptible for \textit{Cj}. as three-week old birds (CAWTHRAW u. NEWELL 2010).

All three isotypes, IgM, IgA and IgY are detectable in chicken after challenged with \textit{Cj}. Both, local and systemic antibodies may contribute to the humoral immunity after \textit{Cj}. colonisation (CAWTHRAW et al. 1994).

2.2.2.4 Cell-mediated immune response

From other enteric bacteria such as \textit{Salmonella} it is known that they trigger an increase of T cells in the caecum. An increase in TCRγδ, CD4+, CD8α+ and CD8β+ T cells can be detected by immunohistochemistry in the caecal mucosa, which peaked at four dpi after \textit{Salmonella} infection. This observation was confirmed by flow cytometric analysis of peripheral blood leukocytes (PBL’s) and spleen leukocytes which suggest that both, the local and systemic cell-mediated immune response may influence the host-\textit{Salmonella} interaction (BERNDT et al. 2007).
There is still a lack of knowledge on the interaction between the cell-mediated immune response and *Campylobacter* in chicken. However, JENNINGS *et al.* (2011) observed an increase in CD3+ T cells in livers of *Cj.* – positive broilers (JENNINGS *et al.* 2011).
3 Goals and objectives

The goal of the study was to understand more about the induction of humoral and cell-mediated immune reactions in chicken after *Cj.* inoculation, and possibly identify *Cj.* strain specific variations in these responses.

A total of five experiments were conducted.

In experiment 1-4 our objectives were

1) To compare the colonisation pattern of *Cj.* strains of human and avian origin in SPF-layer type chicken
2) To analyse the local and systemic immune response after inoculation of these strains

In experiment 5 our objectives were

1) To determine the influence of the genetic background of the chicken on *Cj.* inoculation by comparing broiler and SPF-layer type chicken
2) To identify early immune reactions within the first week after *Cj.* inoculation with two selected *Cj.* strains
4 Material and methods

4.1 Experimental design

A total of five experiments were conducted to investigate colonization properties of *Cj* strains in the chicken and possible immune responses of the host. Table 4 shows all performed experiments including the days of necropsy and number of birds investigated.

All birds were tested negative for *Campylobacter* by cloacal swabs at the day of *Cj* inoculation.

Table 4: Performed experiments
including groups per experiment, number of birds per group and day of necropsy

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Groups per experiment</th>
<th>Number of birds/group</th>
<th>Number of birds/ necropsy day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>24</td>
<td>3 dpi 7 dpi 14 dpi 21 dpi</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>24</td>
<td>6 6 6 6</td>
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<tr>
<td>3</td>
<td>3</td>
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<td>24</td>
<td>6 6 6 6</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>20</td>
<td>5 5 5 5</td>
</tr>
</tbody>
</table>

dpi = days post inoculation

4.1.1 Experiments 1 to 4

In each experiment two groups of 24, in experiment 3 only 20, randomly assigned three-week-old SPF chicken were inoculated with a dose of approximately $10^4$ colony forming units by crop installation with the respective *C. jejuni* strain as shown in Table 5. Each of the four experiments was accompanied by a control group also consisting of 24 (20 in experiment 3) birds. The control group was inoculated with diluent.

At three, seven, 14 and 21 days post inoculation (pi) 5-6 birds per group were randomly selected and sacrificed. Serum samples were collected for detection of
Cj.-specific antibodies by ELISA. After killing and bleeding, individual bodyweight was determined and post-mortem examination was conducted. Liver samples and caecal content were taken to determine the Cj. load.

Parts of the medial caecum and jejunum were removed aseptically for immunohistochemical and histological analysis. Additionally liver samples were taken for histological investigations in experiment 3 and 4.

For detection of IL-6 and IFN-γ mRNA-expression by qRT-PCR samples from spleen, caecum and jejunum were obtained and frozen at -80°C in TRI-Fast GOLD reagent (Peq-Lab Biotechnology GmbH, Erlangen, Germany) until further analysis.

Lymphocytes were isolated from spleen, caecum and jejunum in experiment 1 to 3 for quantification of T cell subpopulations by flow cytometric analysis. In experiment 4 only caecum was dissected for flow cytometric investigation.

At day 21 pi all experiments were terminated.

### 4.1.2 Experiment 5

In the fifth experiment three-week-old commercial broilers and SPF laying type chicken were compared for their susceptibility for Cj. and possible induced immune responses after bacterial inoculation.

Broilers and layers were randomly assigned to three groups each of 20 birds.

Of each genetic background one group was inoculated with the human B2 strain, one group with the avian strain 0097 derived from a laying hen, and the third group served as the C. jejuni - free control and was inoculated with diluent.

On day one, two, three and eight pi five birds per group were randomly selected and sacrificed. After killing and bleeding the bird’s bodyweight was determined.

Swabs of liver and caecum were taken and investigated for Cj. by direct plating.

Additionally caecal content was analyzed for the number of Cj.-colony forming units per gram caecal content.

Medial caecum was dissected aseptically and processed for immunohistochemical investigation of different T cell populations.

Caecum samples were placed in TRI-Fast GOLD reagent for further cytokine detection (IL-6, IFN-γ) by qRT-PCR.
Caecal lymphocytes were isolated and processed for flow cytometric analysis of different T cell populations. At day eight post inoculation the experiment was terminated.

4.2 Bacterial strains

Seven *Campylobacter jejuni* strains, including four of human and three of avian origin were used (Table 5). The strains, except for one, were stored at -70°C with the Cryobank™ system (MAST® Diagnostica GmbH, Reinfeld, Germany). The avian strain of the serogroup Lior6 was stored in skimmed milk at -70°C. The human B2 strain was kindly provided by the Department of Medical Microbiology, University of Göttingen, Germany. The human reference strains 81-176 and NCTC 11168 were kindly provided by the Institute for Microbiology and Hygiene at the Charité, Berlin, Germany. Both the non-toxin producing human strain 84/02 and the two avian strains (0097, av518) were kindly provided by the Friedrich-Loeffler-Institute, Jena, Germany. The avian strain of the serogroup Lior6 derived from the strain collection of the Clinic for Poultry, University of Veterinary Medicine Hannover, Germany.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>strain</th>
<th>original host</th>
<th>Abbrevation used in the text</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B2</td>
<td>human</td>
<td>hu 1</td>
</tr>
<tr>
<td>1</td>
<td>81-176</td>
<td></td>
<td>hu 2</td>
</tr>
<tr>
<td>2</td>
<td>11168</td>
<td>human</td>
<td>hu 3</td>
</tr>
<tr>
<td>2</td>
<td>Lior6</td>
<td>chicken</td>
<td>av 1</td>
</tr>
<tr>
<td>3</td>
<td>J 0097</td>
<td>laying hen</td>
<td>av 2</td>
</tr>
<tr>
<td>3</td>
<td>av518</td>
<td>turkey hen</td>
<td>av 3</td>
</tr>
<tr>
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<td>84/02</td>
<td>human</td>
<td>hu 4</td>
</tr>
<tr>
<td>5</td>
<td>B2</td>
<td>human</td>
<td>hu 1</td>
</tr>
<tr>
<td>5</td>
<td>J 0097</td>
<td>laying hen</td>
<td>av 2</td>
</tr>
</tbody>
</table>

4.3 Animals and housing

Commercial broiler chicks were obtained (BWE-Brüterei Weser-EMS GmbH & Co. KG, Visbek, Rechterfeld, Germany) at day of hatch and raised under isolated
conditions at the Clinic for Poultry, University of Veterinary Medicine Hannover, Germany.

Eggs of specific pathogen-free layer type chicken (Valo® Lohmann-Tierzucht, GmbH, Cuxhaven, Germany) were incubated and hatched at the Clinic for Poultry at the University of Veterinary Medicine Hannover, Germany. The birds were kept in different isolation units under underpressure conditions to avoid contamination. Commercial feed and water were provided ad libitum and the birds were observed daily for clinical signs. All animal experiments were conducted in accordance to the Animal Welfare regulations. The study was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (file reference: 33.12-42502-04-10/0077)

4.4 Preparation of the \textit{Cj.} inocula

The frozen strains were thawed and plated on charcoal cefoperazone deoxycholate agar (CCDA, Oxoid®, Basingstoke, England) for 48 hours under microaerophilic conditions (10% CO$_2$, 5% O$_2$, 85% hydrogen) at 38°C. After two days, one colony of \textit{Cj.} was transferred into 3ml Standard-I-Bouillon (Merck, Darmstadt, Germany) and incubated for another 48 hours under microaerophilic conditions at 38°C to increase bacterial growth.

In prior tests the amount of \textit{Cj.} per ml Standard-I-Bouillon was determined by CfU count. In each case an amount of $10^8$ CfU was ascertained. One ml of the bacterial suspension was diluted with sterile phosphate buffered saline (PBS) to achieve the designated dose for the inoculation of birds. For confirmation of the CfU of \textit{Cj.} in the inocula the bacterial suspensions were serially diluted in a 10-fold dilution series, plated on CCDA plates and incubated for 48 hours under microaerophilic conditions at 38°C. After incubation the grown colonies were counted to calculate the CfU (SMITH et al. 2008).
4.5 Microbiology

4.5.1 Quantitative detection of Cj. in liver and caecal content

At necropsy one caecum and the left liver lobe were dissected from each bird under sterile conditions. One gram of caecal content and liver per animal was filled up to 10 grams with PBS. Subsequently caecal content and liver were homogenized with an ultra-turrax (T25 basic, IKA®-Werke GmbH, Staufen, Germany) at 13.000 rpm for 30 seconds or one minute, respectively. Afterwards the samples were diluted in sterile PBS in a 10-fold and 4-fold dilution series, respectively, and plated on CCDA plates (Oxoid®, Basingstoke, England). After incubation for 48 hours at 38°C under microaerophilic conditions the numbers of CfU/g were calculated based on total bacterial count (SMITH et al. 2008).

4.5.2 Qualitative detection of Cj. in liver and caecum

During necropsy a small incision was made into the right lobe of the liver and the central caecum to reach the caecal mucosa. Swab samples were collected with a one-way-loop and directly plated on CCDA plates (Oxoid®, Basingstoke, England). Afterwards the plates were incubated for 48 hours at 38°C under microaerophilic conditions. Plates were evaluated for being positive or negative for Cj.

4.6 Histology

In experiments 1 to 4 samples of liver, medial caecum and medial jejunum were collected and fixed in phosphate-buffered formalin (4%) for 24 hours at 4°C. Prior to dehydration the samples were washed under running tab water for one hour. Afterwards, dehydration was performed at room temperature in an automatic tissue embedding machine (Citadel 1000, Shandon, Frankfurt, Germany) using an ascending series of isopropanol and acetone.

- Isopropanol 50% for 60 minutes
- 70% for 60 minutes
- 80% for 60 minutes
90% for 60 minutes
100% for 60 minutes
100% for 60 minutes
- Two-times isopropanol/acetone (1:1) for 90 minutes
- Two times acetone for 60 minutes
- Followed by two-times paraffin for 60 minutes

Finally the samples were blocked in paraffin with an automatic tissue blocking machine (Histocenter, Shandon, Frankfurt, Germany) and cooled down.

Prior to cutting, the blocks were placed in a freezer at -20°C. Sections of a thickness of 2 μm were cut with a microtome (model 2040 Autocut, Reichert-Jung, Cambridge Instrument GmbH, Nußloch). Slices were placed in a water bath (40°C), then collected with a glass slide (Thermo Scientific Menzel-Gläser, Braunschweig, Germany) covered with serum-glycerine (3T 013, Division Chroma® Waldeck GmbH &Co. KG, Münster, Germany).

Finally the slides were dried at room temperature before being stained with hematoxylin-eosin (HE) in an automatic staining machine (Varistain 24-2, Shandon, Frankfurt, Germany).

HE staining was performed according to following procedure:
   a) Deparaffinization and dehydration:
      a. two-times xylene for 5 minutes
      b. a descending row of isopropanol for 5 minutes each
         i. 100%, 96%, 70%, 50%, 20%
      c. washing with distilled water for 5 minutes
      d. staining with hematoxylin for 10 minutes
      e. washing under running tab water for 10 minutes
      f. eosin staining (1% eosin) for 5 minutes
      g. two-times washing with distilled water for 30 seconds each
   b) Dehydration:
      a. ascending row of isopropanol
         i. 70 % for 3 minutes ; 96% for 3 minutes ; 100% for 5 minutes
      b. carbol-xylene for 5 minutes
c. xylene for 5 minutes

d. xylene for 10 minutes

Finally the slides were covered with Aquatex®, Merck, Darmstadt, Germany.

All slides were blinded and investigated for histopathological lesions such as infiltration of leukocytes, ballooning cells or degenerative changes. Intestinal samples were additionally monitored for crypt abscesses or villi degeneration as previously described (MORALES et al. 2011; MURPHY et al. 2011).

4.7 Isolation of leukocytes and flow cytometric analysis

In experiments one to three splenic and intestinal IEL’s were isolated according to established protocols (SCHWARZ et al. 2011).

4.7.1 Isolation of spleen cells

The splenic capsule was removed and the spleen was mashed with PBS through a 70 μm nylon cell strainer (BD Falcon®, Heidelberg, Germany). After centrifugation at 1500 rpm for 10 minutes at 4°C the splenic leukocytes were isolated by density centrifugation using Biocoll separating solution (1.09 g/ml ; Biochrom AG, Berlin, Germany) as previously described (LIMAN u. RAUTENSCHLEIN 2007). For collection of leukocytes at the interphase, 10 ml of the cell suspension was underlayed carefully with 7 ml of Biocoll and the interphase was harvested after centrifugation at 3300 rpm for 10 minutes at room temperature. Prior to use, Biocoll has to be warmed at room temperature and be kept in the dark. Afterwards the samples were washed twice with PBS via centrifugation at 1200 rpm, for 10 minutes at 4°C. Finally the cells were resuspended in 1 ml of FACS-buffer and kept at 4°C before counting.

4.7.2 Isolation of intraepithelial lymphocytes

One caecum, excluding the caecal tonsil, and the jejunum were dissected aseptically and stored in PBS on ice for further processing.
The gut sections were cut open longitudinally and washed with PBS. The sections were cut into small pieces of approximately 1 cm length and digested in PBS containing EDTA at a concentration of 0.925 mg of EDTA per 5 ml of PBS for caecum and 1.85 mg of EDTA in 50 ml of PBS for jejunal samples. The samples were stirred for 30 minutes at 37°C for digestion. The caecum samples then were filtered through a 70 μm cell strainer implemented with 20 mg of nylon wool fibre (Polyscience Inc., Eppelheim, Germany). Cells were centrifuged at 1600 rpm for five minutes at room temperature, resuspended in 1 ml PBS containing 1% bovine serum albumine (BSA; PAA Laboratories Company, Pasching, Austria; pH 7.0) and kept at 4°C till further use. After digestion each sample of the jejunum was transferred into a 50 ml tube and centrifuged once at 1200 rpm for 5 minutes at 4°C. Afterwards each sample was resuspended in 40 ml of PBS and centrifuged again as described before. Subsequently each sample was filtered through a column of a 5ml syringe filled with 340 mg of nylon wool fibre (Polyscience Inc., Eppelheim, Germany). Thereafter the suspensions were centrifuged at 1200 rpm at 4°C for 5 minutes and the cells were resuspended with 10 ml of PBS. Isolation of leukocytes was performed by density centrifugation using Biocoll separating solution (Biochrom AG, Berlin, Germany) as previously described (GÖBEL 2000). For that, the 10 ml cell suspension was carefully underlayed with 7 ml of Biocoll and centrifuged at 2000 rpm for 10 minutes at room temperature. Cells were than harvested from the interphase, washed twice in PBS, resuspended in 1 ml FACS-buffer and stored at 4°C before counting.

4.7.3 Cell counting

Each sample was diluted 1:10 in Trypan Blue Stain 0.4% (Gibco®, Invitrogen cooperation, Darmstadt, Germany). The isolated leukocytes were counted in a Neubauer Zählkammer. The cell number was than calculated using the following formula:

\[
\text{Cell count/ml} = \text{number of cells} \times \text{fields counted (1, 5, 25)} \times 10 \ (\text{pre-dilution}) \times 10^4 \ (\text{chamber volume})
\]
For further use samples were adjusted to the same cell number by diluting in FACS-buffer.

**4.7.4 Flow cytometric analysis**

The relative numbers of CD3+ lymphocytes were determined using the Beckman Coulter Epics XL© flow cytometer (Beckman Coulter, Krefeld, Germany) and data were than analyzed with the EXPO 32 ADC program.

The lymphocyte population was gated depending on size and granularity according to the forward and sideward scatter characteristic. For each triple staining combination a protocol was created.

Unstained cells and cells stained with one dye served as controls. They were used to adjust settings and for compensation prior to the measurement of multi-colour samples as given in Table 6.

Each time 200,000 events per intestinal sample and 10,000 events per spleen sample were measured.

The staining set-up for T cell analysis is given in Table 6.

**Table 6: Staining set-up for flow cytometric analysis**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sample</th>
<th>Staining together with</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 to 4</td>
<td>caecum and jejunum</td>
<td>CD3+ CD8α+ and CD4+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8α+ and TCRα/β+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8α+ and TCR γ/δ+</td>
<td></td>
</tr>
<tr>
<td>Experiment 1 and 2</td>
<td>spleen</td>
<td>CD8α+ and CD4+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8α+ and TCRα/β+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8α+ and TCR γ/δ+</td>
<td></td>
</tr>
<tr>
<td>Experiment 5</td>
<td>caecum</td>
<td>CD3+ CD8α+ and CD4+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8α+ and TCRα/β+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD8α+ and TCR γ/δ+</td>
<td></td>
</tr>
</tbody>
</table>

CD = cluster of differentiation. TCR = T cell receptor. { = with either cominate
4.8. Immune cell investigation

4.8.1 Antibodies

Before use all antibodies were titrated to determine the optimal concentration for the staining procedures. Table 7 gives an overview of the used antibodies.

Table 7: Monoclonal antibodies, and their respective concentration used for flow cytometric and immunohistochemical analysis

<table>
<thead>
<tr>
<th>Method</th>
<th>Monoclonal antibodies</th>
<th>conc.</th>
<th>working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/ml</td>
<td>μg/ml</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Mouse anti-chicken CD3-RPE, Clone CT-3</td>
<td>0.1</td>
<td>1</td>
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<tr>
<td></td>
<td>Mouse anti-chicken CD4-FITC, Clone CT-4</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-chicken TCRαβ-FITC, Clone TCR-2</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-chicken TCRγδ-FITC, Clone TCR-1</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-chicken CD8α- Biot, Clone CT-8</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Streptavidin-SPRD</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Mouse anti-chicken CD4 UNLB, Clone CT-4</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-chicken CD8β UNLB, Clone EP42</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Mouse anti-chicken KUL-01 UNLB</td>
<td>0.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

RPE= phycoerythrin, FITC= fluorescin isothiocyanate, Biot= biotinylated, SPRD= spectral red, UNLB= unlabelled

4.8.2 Immunohistochemistry

For the immunohistological investigation of immune cells the centrical caecum was dissected from each bird, wrapped in aluminium foil, snap frozen in liquid nitrogen and stored at -80°C till further processing as previously described (VERVELDE et al. 1996; BERNDT et al. 2007).

The frozen caecum samples were fixed on the block with freezing medium (Jung, Leica Microsystems Nussloch GmbH, Heidelberg, Germany) and cut into slices of a thickness of 8 μm with a Cryostat (Jung, Frigocut 2800E, Leica GmbH, Bensheim, Germany). The slices were placed on slides (Thermo Scientific, Superfrost Ultra Plus®, Braunschweig, Germany). Until final staining, slides were kept in an incubator containing silica gel (Kraemer & Martin GmbH, St. Augustin, Germany) for drying. For staining the slides were first fixed with ice-cold acetone for 10 minutes at 4°C followed by a drying step at room temperature for 10 minutes. After drying the slides were washed in PBS for 10 seconds. For inactivation of the endogenous peroxidase in
the samples the slides were placed for 15 minutes in a coplin jar containing 0.03 % H$_2$O$_2$. The slides were washed twice with PBS for 10 seconds and 5 minutes subsequently. During the following steps each slide was always covered with 500 μl of the respective solution.

Afterwards a blocking antibody, in addition with Avidin D was added according to manufacturer’s instructions (Vectastain®, Elite ABC Kit, Vector Laboratories Inc., Burlingame, USA) and the slides were incubated for 20 minutes at room temperature. After the blocking step the primary antibody in addition with Biotin was added and incubated for one hour in an incubator at 37°C.

In the first four experiments, T cells were detected by using mouse anti-chicken unlabelled monoclonal antibodies (anti-CD4 and anti-CD8β; 0.5mg/ml; Southern Biotech, provided by Biozol) at a dilution of 1:700 and 1:1000, respectively. In the fifth experiment the monoclonal antibodies anti-CD4, anti-KUL-01 and anti-CD8β were used at a dilution of 1:700, 1:700 and 1:500, respectively. An overview of the used antibodies and their appropriate concentration is given in Table 6.

After following washing steps for 10 seconds and for 5 minutes with PBS the secondary anti-mouse IgG biotinylated antibody was added and the slides were incubated for 30 minutes at room temperature. Afterwards another washing step in PBS was performed and, than the ABC-reagent (Vectastain® Elite® ABC Kit; Vector Laboratories Inc., Burlingame, CA 94010, USA) was added for another 30 minutes at room-temperature. Finally, after another washing step freshly prepared 3,3′-diaminobenzidine peroxidase substrate (DAB ; Vector Laboratories Inc.) was placed on the slides till a brown colouration appeared. After a 10 minute washing step the slides were counter-stained with hematoxylin (Linaris, Wertheim, Germany) for 10 seconds. Finally the slides were kept for 5 minutes under running tap water before being covered with Aquatex®, Merck, Darmstadt, Germany and a cover glass.

The slides were blinded and evaluated by counting the number of positive stained cell populations within three crypts in the lamina propria and epithelium of five representative microscopic fields at 200x magnification (SCHWARZ et al. 2011).
4.8.3 Staining procedure for flow cytometric analysis

In experiments 1 to 4, 10^6 IEL’s of the jejunum and caecum were triple stained with a RPE-conjugated mouse anti-chicken-CD3 (1:100) and a biotinylated mouse anti-chicken-CD8α (1:100) and additionally with a FITC-labelled mouse anti-chicken CD4, TCRqβ or TCRγδ (1:100) for each combination. Prior to use the mouse anti-chicken-CD8α was conjugated with streptavidin using a SpectralRed™ (SPRD) antibody (5 μl). All antibodies were obtained from Southern Biotech, provided by Biozol, Echingen, Germany (SCHWARZ et al. 2011).

Double staining of 10^6 splenic lymphocytes was conducted in experiment 1 and 2 using the biotinylated mouse anti-chicken-CD8α (1:100) antibody in combination with a FITC-labelled mouse anti-chicken CD4, TCRqβ or TCRγδ respectively (1:100). Again, prior to use, the biotinylated mouse anti-chicken-CD8α had to be conjugated with streptavidin using SpectralRed™ (SPRD) (5 μl).

In experiment 5, 10^6 IEL’s of each caecum sample were stained with the RPE conjugated mouse anti-chicken-CD3 (1:100) antibody. In addition FITC-labelled mouse anti-chicken CD4, TCRqβ or TCRγδ antibodies were added respectively (1:100). Before staining the cells were plated in a round-bottom 96-well plate and centrifuged for four minutes at 4°C for 2000 rpm. Supernatants were discarded and the cell pellets were re-suspended with 100μl FACS buffer containing the corresponding monoclonal antibodies as given in Table 6. After an incubation period of 30 minutes at 4°C in the dark, cells were washed twice with 100μl of FACS buffer by centrifugation at 2000 rpm for five minutes.

4.9 Real-time quantitative RT-PCR

Total RNA was isolated from spleen, jejunal and caecal samples by using 1000 μl TRI-Fast GOLD reagent (PeqLab, Biotechnologie GmbH, Erlangen, Germany) according to manufacturer’s instructions.

For isolation the samples were thawed and homogenized with the PEQLAB Tissue homogenizer Precellys®. The tubes contained 1 ml of TriFast (PeqLab, Erlangen, Germany) and Precellys ceramic beads (1.4 mm, PeqLab, Erlangen, Germany).
The following tissue homogenizing protocols were used:
- for spleen
  - 5500 rpm - 1x20 sec
- for intestine
  - 6800 rpm - 1x30 sec - 0.05 > 5 min break > 6800 rpm - 1x30 sec - 0.05

After homogenization, the samples were incubated for 5 minutes at room temperature. Subsequently, 200 µl of Chloroform (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) per sample were added and shaken for 15 seconds. After incubation at room temperature for 10 minutes the samples were centrifuged at 12,000 x g, and 7°C for 5 minutes. Afterwards 500 µl of Isopropanol (AppliChem, Biochemica, Darmstadt, Germany) and the liquid phase of the samples were transferred to a tube and mixed. After incubation on ice for 12 minutes another centrifugation was performed with 12,000 x g at 4°C for 10 minutes. Supernatants were discarded and 1 ml of ethanol (75%; AppliChem, Biochemica, Darmstadt, Germany) was added twice for washing the pellet.

The pellet was dried at room temperature for 10 minutes. Finally, it was dissolved in 50 µl of RNA’se/DNA’se free water (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) under gentle mixing on the rocking table (Biosan T100 Thermo Shaker, Biodeal, Markkleeberg, Germany) at 58°C and 250 rpm for 10 minutes.

RNA concentration and quality was determined using the NanoDropND-1000 (PeqLab, Biotechnologie GmbH).

Primers and Probes for chicken IL-6, IFN-γ and house-keeping gene 28S have been described previously and are presented in Table 8 (SMITH et al. 2005; SCHWARZ et al. 2011). Real-time quantitative RT-PCR was performed using the Ambion AgPath-ID One-Step RT-PCR kit (Life Technologies, Carlsbad, USA). Amplification and quantification of specific products was conducted by the Mx3005PTM thermal cycle system and Mx3005PTM Q PCR Software (STRATAGENE; Agilent Technologies Company). The following cycle profile was applied:
One cycle at 50°C for 30 min and 95°C for 10 min, and 40 cycles at 95°C for 20 sec. and 60°C for 1 min.
The threshold cycle ($C_t$) values of the samples were normalized to the 28S $C_t$ values by using following formula (POWELL et al. 2009).

$$C_t + (N_t' - C_t) * S/S'$$

$C_t$ = cycle threshold  
$C_t'$ = mean $C_t$ for 28S RNA in the samples  
$N_t'$ = mean $C_t$ for 28S RNA among all samples  
$S$ = slopes of the regressions of the standard plots for the test mRNA  
$S'$ = slopes of the regressions of the standard plots for the 28S RNA

Table 8: Primers and probes for qRT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Probe / Primer</th>
<th>Sequence (5'-3')</th>
<th>Accession No(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>Probe</td>
<td>HEX-AGGACCCTACGGACCTCCACCA-BHQ2</td>
<td>X59733</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GGCGAAGCCAGAGGAAACT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GACGACCATTTCACGTC</td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Probe</td>
<td>FAM-AAGCTCCCCAGAGACGTGTTGA-TAMRA</td>
<td>Y07922</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GTGAAAGATATCATGGACCTGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TTCTGTAAGATGCTGAAGACGATTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>FAM-AGGAGAAAATGCGCTGACAGCTCTCCA-TAMRA</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Probe</td>
<td>GGCTCGCCGGCTTCGA</td>
<td>AJ250838</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGTAGGCTGAAAAGGCGAAGC</td>
<td></td>
</tr>
</tbody>
</table>

F: Forward primer, R: Reverse primer, \(^a\) Refers to genomic DNA sequence in Gene Bank

4.10 Enzyme-linked immunosorbent assay (ELISA)

Serum samples were tested for IgG-type and IgM-type specific anti-$C_{j}$. antibodies using a biotin-streptavidin ELISA described previously (HAAS et al. 1999). 100μl/well of $C_{j}$. antigen (1 μg/ml), derived from soluble bacterial proteins in coating-puffer was adsorbed to ELISA plates (Polystren-microtiterplate, Maxisorb F96, Nunc, Wiesbaden, Germany) for one hour at 37°C on a rocking table. The used antigen was obtained from the $C_{j}$. strain Lior6 available at the Clinic for Poultry, Hannover, Germany had been prepared previously.
The strain has been grown on Columbia sheep blood agar (Oxoid®, Basingstoke, England) and finally flushed with PBS. To receive soluble bacterial protein the bacterial suspension was centrifuged for 20 minutes at 6.700 \( x \) g and the bacterial pellet was resuspended in PBS following two washing steps with coating-puffer. After incubating overnight at 4°C the suspension was sonicated three times for 30 seconds. Bigger bacterial particles were removed by centrifugation at 40.000 \( x \) g for 30 minutes. The clear supernatant was harvested and stored at -70°C partitioned in 2 ml.

For antibody detection serum samples were diluted 1:100 in PBS + Tween20 (0.05%; Bio-Rad, München, Germany) and 100μl/well were added in duplicates after the plates had been washed three times with PBS+Tween20 (0.05%). A prior blocking step was not performed, due to the use of Tween20 as an ingredient in PBS, which is suggested to make other blocking substances needless.

The samples were incubated for 15 minutes at 37°C on the rocking table followed by three washing steps. For IgG detection, first, 100μl of biotinylated rabbit anti-chicken-IgG (Dianova, Hamburg, Germany) diluted 1:40.000 in PBS + Tween20 (0.05%) was added to each well followed by a 15 minute incubation period on the rocking table at 37°C. After three washing steps, 100μl of streptavidin-peroxidase (SA-PO) (Dianova, Hamburg, Germany), diluted 1:40.000 in PBS + Tween 20 (0.05%) was added to each well also followed by an incubation for 15 minutes at 37°C on the rocking table. For detection of IgM-type antibodies a directly conjugated antibody (goat anti-chicken-IgM) was used (Bethyl, Montgomery, Texas, USA). The antibody was diluted in PBS + Tween20 (0.05%) at 1:40.000 and incubated for 15 minutes at 37°C on the rocking table. For IgM-detection the conjugation step with SA-PO was excluded.

For colour development 100 μl of \( \text{H}_2\text{O}_2 \) activated 2,2’-Azinobis-[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) (Boehringer, Manneim, Germany) were added per well. After 15 minutes incubation at 37°C 50% methanol was added to stop the colour change reaction.

OD-values were measured at 405 nm using an spectrophotometer (Magellan V 6.4 ; Tecan® sunrise).
The cut-off was defined by the mean OD-value of all non-inoculated birds plus two-times standard deviation of all non-inoculated birds and accounted 0.193 for IgG and 0.243 for IgM.

4.11 Statistical analysis

All data are expressed as mean per group ± standard deviation. The statistical analyses were performed using the Statistix® version 9.0 (Analytical software, Tallahassee, FL, USA).

For statistical analysis of differences Kruskal-Wallis All-Pairwise Comparisons Test ($P < 0.05$) was used to compare T lymphocyte subsets in the IEL’s as well as for differences between cytokine levels of different groups. For differences in the immune cell subsets in the lamina propria the analysis of variance ($P < 0.05$) was used.
5 Results

For better understanding of the abbreviations used for the different \( Cj. \)-strains in the conducted experiments, Table 5 is shown again.

Table 5: Overview of tested \( Cj. \) strains

<table>
<thead>
<tr>
<th>Experiment</th>
<th>strain</th>
<th>original host</th>
<th>Abbreviation used in the text</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B2</td>
<td>human</td>
<td>hu 1</td>
</tr>
<tr>
<td></td>
<td>81-176</td>
<td>human</td>
<td>hu 2</td>
</tr>
<tr>
<td>2</td>
<td>11168</td>
<td>human</td>
<td>hu 3</td>
</tr>
<tr>
<td></td>
<td>Lior6</td>
<td>chicken</td>
<td>av 1</td>
</tr>
<tr>
<td>3</td>
<td>J 0097</td>
<td>laying hen</td>
<td>av 2</td>
</tr>
<tr>
<td></td>
<td>av 518</td>
<td>turkey hen</td>
<td>av 3</td>
</tr>
<tr>
<td>4</td>
<td>84/02</td>
<td>human</td>
<td>hu 4</td>
</tr>
<tr>
<td>5</td>
<td>B2</td>
<td>human</td>
<td>hu 1</td>
</tr>
<tr>
<td></td>
<td>J 0097</td>
<td>laying hen</td>
<td>av 2</td>
</tr>
</tbody>
</table>

5.1 Experiments 1 – 4

5.1.1 Effect of \( Cj. \) inoculation on general health aspects

Birds in all inoculated and non-inoculated groups showed a comparable growth development independent of the inoculated \( Cj. \) strain (Figure 3 a,b). Sometimes there was a trend for lower bodyweight gain in \( Cj. \)-inoculated groups, compared to the \( Cj. \)-free controls, but no significant differences were detected.
Figure 3: Mean bodyweight development in gram (± standard deviation) of *Cj*-inoculated and *Cj*-free control SPF-layer-type chicken of Experiment 1 (a) and Experiment 3 (b) as representative experiments. *n* = 5-6 birds per group and day. dpi = days post inoculation. (ANOVA, Tukey *P* ≤ 0.05). Error bars indicate the standard deviation.

No clinical signs or pathological lesions were observed in any of the *Cj*-inoculated nor in the control groups in either experiment.
No significant histological lesions were observed in the jejunal, caecal and liver sections, neither of the *Cj*.-inoculated, nor of the control groups, at any of the investigated time points (Figure 4 a-h).
Figure 4: Histological sections of jejunum (a,c,e) and caecum (b,d,f) at 200-fold magnification of non-inoculated (a,b), hu3-inoculated (c,d) and av1-inoculated (e,f) layer-type chicken as representatives for the conducted experiments. Liver sections of a non-inoculated (g) and an av2 inoculated bird (h) at 100-fold magnification.

5.1.2 Colonisation of chicken by different Cj. strains

5.1.2.1 Cj. isolation from caecal content

The strain hu1 was not detected in caecal content of SPF layer-type chicken between three and 21 dpi. The reference strain hu2 was detected in the caecal content from day seven pi onwards, whereas hu3 and hu4 were found at each investigated time point throughout the conducted experiments. The results are shown in Figure 5 a-d.
Figure 5: Numbers of CfU in caecal content of SPF-layer-type chicken after inoculation with human Cj strains.

All control birds, which had been inoculated with Cj-free diluent remained Cj-free throughout the experiments. One dot represents one bird. \( n = 5-6 \) birds per group / time point. hu = human. n.d. = not done. dpi = days post inoculation. — = indicates the mean. Error bars indicate the standard deviation.

The avian strains av1 and av2 were isolated from caecal content at each investigated time point. The strain av3 was only detected at day 14 and 21 pi. The data for each strain is given in Figure 6 a-c.
Log10 CFU / g caecal content

![Graph showing Log10 CFU / g caecal content over time.]

c) Figure 6: Numbers of CFU in caecal content of SPF-layer-type chicken after inoculation with avian Cj. strains.

All control birds, which had been inoculated with Cj.-free diluent remained Cj.-free throughout the experiments. One dot represents one bird. n = 5-6 birds per group / time. av = avian. n.d. = not done. dpi = days post inoculation. — = indicates the mean. Error bars indicate the standard deviation.

For nearly all strains the Cj. – detection level in the caecal content was between $10^6$ and $10^8$ CFU / g caecal content. Only hu2 showed detection rates of $10^3$ and $10^5$ CFU / g caecal content at day seven and 14 dpi, respectively. The non-Cj.-inoculated controls remained negative in all experiments.

5.1.2.2 Cj. detection in the liver

Liver samples of SPF layer-type chicken inoculated with the Cj. strains hu1 to hu4, av1 and av3 were all Cj. negative in culture.

Only strain av2 was detected in liver homogenate. However, only six birds were Cj.-positive in liver samples and the detection rate was low.

The number of positive birds per day is shown in Table 9 and the detection rate is presented in Figure 7.
Table 9: SPF layer-type chicken tested Cj. positive in liver samples after av2 - inoculation

<table>
<thead>
<tr>
<th>dpi</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of av2 positive birds / group</td>
<td>2/5</td>
<td>1/5</td>
<td>2/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>

Figure 7: Cj. counts from liver homogenates of SPF-layer-type chicken inoculated with the avian strain av2 (Experiment 3)
One dot represents one bird. n = 5 birds per group / time point. dpi = days post inoculation. av = avian.
— = indicates the mean. Error bars indicate the standard deviation.

5.1.3 Effect of Cj. colonisation on humoral immunity parameters

5.1.3.1 Induction of Cj.-specific IgG-type antibodies
Cj.-specific IgG-type antibodies were detected by ELISA.
The hu1, 2 and 4 strains did not induce Cj.-specific antibody levels above the cut-off at any of the investigated time points. The hu3 strain induced low, but above cut-off, levels at 14 and 21 dpi (Figure 8). Cj. non-inoculated birds did not show Cj.-specific IgG-type antibody levels above the cut-off (data not shown).
Figure 8: Group-mean OD values of serum \textit{Cj}.-specific IgG-type antibodies of SPF layer-type chicken at different dpi which had been inoculated with \textit{Cj}. strains hu1 to hu4. \( n = 5-6 \) birds per group / time point. Dotted line shows the cut-off level. dpi = days post inoculation. hu = human. Error bars indicate the standard deviation.

Av1 inoculated birds already showed \textit{Cj}.-specific antibody levels above cut-off at seven dpi and birds were still antibody – positive at day 14 and 21 pi. The av2 and av3 strains induced levels of \textit{Cj}.-specific IgG antibodies above cut-off at day 21 pi (Figure 9). The control groups did not show \textit{Cj}.-specific IgG-type antibody levels above cut-off (data not shown).
5.1.3.2 Induction of *Cj.*-specific IgM-type antibodies

No IgM-type specific anti-*Cj.* antibody levels above the cut-off were detectable throughout the conducted experiments (data not shown).

5.1.4 Effect of *Cj.* colonisation on the cell-mediated immune response

5.1.4.1 Flow cytometric analysis

All analysed spleen samples showed comparative values in the relative percentage of CD4+, CD8+, TCRαβ+ and TCRγδ+ T cells of total lymphoid cells between *Cj.* inoculated and non-inoculated groups at any time point (data not shown).

The analysis of the relative percentage of CD4+, CD8+, TCRαβ+ and TCRγδ+ within the IEL's of caecum and jejunum showed overall no significant differences between *Cj.* inoculated and non-inoculated control groups in any of the experiments.

However, two exceptions were observed. On day three pi the relative mean percentage of intraepithelial CD3+CD4+ T lymphocytes was significantly decreased
\((P \leq 0.05)\) in the jejunum of the hu1 inoculated group compared to the non-inoculated control group (Figure 10).

Figure 10: Comparison of the relative percentage of intraepithelial CD4+ T cells of the jejunum after inoculation of SPF-layer-type chicken with diluent, hu1 and hu2 (Exp. 1) at three and seven days post inoculation. \(n = 6\) birds per group and day. Different letters indicate significant differences between groups at indicated time points (\(P \leq 0.05\) Kruskal-Wallis-Test). dpi = days post inoculation. CD4+ IEL are expressed as the percentage of CD3+ T cells. Error bars indicate the standard deviation.
Birds inoculated with the hu3 strain showed a significant increase of intraepithelial TCRαβ+ T lymphocytes ($P \leq 0.05$) in caecum samples at 14 dpi compared to non-inoculated control birds (Figure 11).

![Figure 11](image)

**Figure 11:** Comparison of the relative percentage of intraepithelial TCRαβ+ T cells in caecum samples of SPF-layer-type chicken after inoculation with diluent, hu3 or av1 (Exp. 2) at seven and 14 days post inoculation. $n = 6$ birds per group and day. Different letters indicate significant differences between groups at indicated time points ($P \leq 0.05$ Kruskal-Wallis-Test). dpi = days post inoculation. TCRαβ+ IEL are expressed as the percentage of CD3+ T cells. Error bars indicate the standard deviation.

5.1.4.2 Immunohistochemical investigation

No significant differences in the numbers of the investigated T cell populations (CD4+, CD8β+) in the lamina propria of the caecum were observed between *Cj.* inoculated and non-inoculated SPF-layer-type chicken (Figure 12 a,b).
5.1.4.3 Cytokine expression

5.1.4.3.1 IFN-γ expression

A significant increase of IFN-γ was observed at day three pi in caecum samples of SPF-layer-type chicken which had been inoculated the av1 strain. On the other hand, hu4 showed a significant IFN-γ decrease at day three pi compared to the diluent inoculated groups ($P \leq 0.05$). Additionally, no IFN-γ expression was measured in the caecum for hu3 and av2 inoculated SPF-layer-type chicken. Inoculation with hu1 and hu2 did not induce any IFNγ expression in caecal samples (Figure 13 a-d).
Figure 13: Quantification of IFN-γ mRNA expression in caecum samples of SPF-layer-type chicken
inoculated with the respective C. jejuni strain (a): Experiment.1, (b): Experiment.2, (c): Experiment.3, (d): Experiment.4). n = 5 birds / group. Data corrected for variation by 28S mRNA levels and presented as 35-Ct in mRNA expression of Cj - and diluent inoculated birds. Letters indicate significant differences between groups (Kruskal-Wallis-Test $P \leq 0.05$). dpi = days post inoculation. Presented is the group mean. Error bars indicate the standard deviation.

5.1.4.3.2 IL-6 expression

The av1 and av3 strain induced a significant increase of IL-6 in caecum samples on day three pi ($P \leq 0.05$). For hu3 and hu4 no IL-6 expression were measured in caecum samples. No differences were found for the other investigated strains compared to the diluent inoculated groups (Figure 14 a-d).
Figure 14: Quantification of IL-6 mRNA expression in caecum samples of SPF-layer-type chicken infected with the respective *C. jejuni* strain in the four conducted experiments (a): Exp.1, (b): Exp.2, (c): Exp.3, (d): Exp.4). n = 5-6 birds / group. Data corrected for variation by 28S mRNA levels and presented as 35-Ct in mRNA expression of *Cj* - and diluent inoculated birds. Letters indicate significant differences between groups (Kruskal-Wallis-Test *P* ≤ 0.05). dpi = days post inoculation. Presented is the group mean. Error bars indicate the standard deviation.

5.2 Experiment 5

In the fifth experiment both, SPF-layer-type chicken and commercial broilers were inoculated with the hu1 and the av2 *Cj*. strains.

5.2.1 Effect of *Cj*. inoculation on general health aspects

No clinical signs or pathological lesions were observed in any of the *Cj*. – inoculated nor in the diluent inoculated control groups.

The SPF-layer-type chicken in all inoculated and non-inoculated groups showed a comparable growth development independent of the inoculated *Cj*. strain.

Av2 inoculated broilers showed a significant reduced weight at day one post inoculation compared to the control and hu1 inoculated group. There were no differences at the other investigated time points (Figure 15 a,b).
Figure 15: Mean bodyweight development in gram of SPF-laying-type chicken (a) and commercial broilers (b) inoculated with Cj. strains hu1 and av2. n = 5 birds / group and day. dpi = days post inoculation. Different letters indicate significant differences between groups at indicated time points (ANOVA, Tukey $P \leq 0.05$). Error bars indicate the standard deviation.
5.2.2 Colonisation pattern of hu1 and av2 in SPF layer-type chicken and commercial broilers

5.2.2.1 Detection of Cj. in caecal content

The hu1 inoculated SPF-layer-type chicken remained Cj. negative till the end of the experiment at day eight pi. On the other hand, at day two, three and eight pi three, four and three broilers were hu1-positive in caecal content, respectively (Figure 16 a,b)

Figure 16: Mean CFU in the caecal content of a) layer-type chicken and b) broilers inoculated with the Cj. strain hu1 (Exp. 5). One dot represents one bird. n = 5 birds per group / time point. dpi = days post inoculation. — = indicates the mean. Error bars indicate the standard deviation.

SPF-layer-type chicken were negative for the av2 strain at day one pi. At day two and at day three pi one and three birds per group were tested av2-positive in the caecal content, respectively. At the end of the experiment on day eight all five investigated laying-type chicken were Cj. - positive in the caecal content.

Av2 was detectable at day one pi in the caecal content in one broiler. At day two pi four broilers and afterwards all five birds per group were av2-positive in the caecal content (Figure 17 a,b).
Figure 17: Mean CFU in the caecal content of a) layer-type chicken and b) broilers inoculated with the *Cj*. strain av2 (Exp. 5). One dot represents one bird. n = 5 birds per group / time point. dpi = days post inoculation. — = indicates the mean. Error bars indicate the standard deviation.

5.2.3.2 Qualitative *Cj*. detection in spleen and liver

Throughout the experiment all spleens of SPF-layer-type and broiler chicken were tested negative for *Campylobacter* in all groups and at all time points.

Table 10 gives an overview of livers tested positive during Experiment 5.

<table>
<thead>
<tr>
<th>Chicken</th>
<th>1 dpi</th>
<th>2 dpi</th>
<th>3 dpi</th>
<th>8 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler</td>
<td>1 / 5</td>
<td>0 / 5</td>
<td>2 / 5</td>
<td>0 / 5</td>
</tr>
<tr>
<td>Layer-type</td>
<td>1 / 5</td>
<td>0 / 5</td>
<td>0 / 5</td>
<td>1 / 5</td>
</tr>
</tbody>
</table>
5.2.3 Effect of *Cj*. colonisation on the cell-mediated immune response

5.2.3.1 Flow cytometric analysis

5.2.3.1.1 CD4⁺ IEL’s

Both, layer-type chicken and broilers inoculated with the hu1 strain, showed no significant differences in the relative percentage of CD3⁺CD4⁺ T cells compared to the non-inoculated control group ($P \leq 0.05$) at any investigated time point. SPF-layer-type chicken inoculated with the av2 strain showed no differences at the level of CD3⁺CD4⁺ T lymphocytes. At day one and two pi av2-inoculated broiler chicken showed a significant decrease in CD3⁺CD4⁺ T lymphocytes in the IEL’s of caecum ($P \leq 0.05$) compared to the control group (Figure 18 a,b).
Figure 18: Comparison of the relative percentage of intraepithelial CD4+ T cells of the caecum after inoculation of SPF-layer-type chicken (a) and broilers (b) with diluent, hu1 and av2. n = 6 birds per group and day. Different letters indicate significant differences between groups at indicated time points ($P \leq 0.05$ Kruskal-Wallis-Test). dpi = days post inoculation. TCRαβ+ IEL are expressed as the percentage of CD3+ T cells. Error bars indicate the standard deviation.

5.2.3.1.2 TCRαβ+ IEL’s

Hu1-inoculated layer-type and broiler chicken showed no significant differences in the relative number of TCRαβ+ IEL’s in the caecum compared to the non-inoculated controls. Av2-inoculated layer-type chicken showed a significant increase in TCRαβ+ IEL’s in the caecum at day one pi ($P \leq 0.05$). This increase was not observed in broilers inoculated with the same strain (Figure 19 a,b).
Figure 19: Comparison of the relative percentage of intraepithelial TCRαβ+ T cells of the caecum after inoculation of SPF-layer-type chicken (a) and broilers (b) with diluent, hu1 or av2. n = 6 birds per group and day. Different letters indicate significant differences between groups at indicated time points (P ≤ 0.05 Kruskal-Wallis-Test). dpi = days post inoculation. CD4+ IEL are expressed as the percentage of CD3+ T cells. Error bars indicate the standard deviation.
5.2.3.1.3 TCRγδ+ IEL’s

Layer-type chicken and broilers, inoculated with either strain hu1 or av2, showed no significant differences in comparison to the non-inoculated group. However, a trend for hu1 inoculated broilers can be seen at two dpi, with significant higher numbers of TCRγδ+ ($P \leq 0.05$) compared to the av2 inoculated group (Figure 20 a,b).
5.2.3.2 Immunohistochemical investigation

The immunohistochemical investigation of the lamina propria included the CD4+, CD8β+ and the monocyte/macrophage-like (KUL-01) cells.

5.2.3.2.1 CD4+ lamina propria leukocytes

Hu1- inoculated layer-type chicken showed an significant increase in CD4+ lamina propria leukocytes (LPL’s) at day one compared to the control group (P ≤ 0.05). The other time points and the group inoculated with the av2 strain showed no differences in the number of CD4+ LPL’s compared to the non-inoculated group.
Both, hu1- as well as av2-inoculated broilers showed a significant increase of CD4+ LPL’s at day one pi compared to the non-inoculated group ($P \leq 0.05$). At the following time points no differences were observed between the inoculated and the non-inoculated groups (Figure 21 a,b).
Figure 21: Mean number of CD4+ lamina propria leukocytes (LPL’s) per three crypts of five microscopic fields at 200 fold magnification in SPF-layer-type chicken (a) and broilers (b). Different letters indicate significant differences between groups (ANOVA, Tukey P ≤ 0.05). n = 5 birds / group and day. dpi = days post inoculation. Error bars indicate the standard deviation.

5.2.3.2.2 CD8β+ lamina propria leukocytes

The hu1-inoculated layer-type chicken showed a significant increase in CD8β+ LPL’s at day one post inoculation (P ≤ 0.05). At the following investigated time points no differences were observed between the hu1 inoculated and the non-inoculated group.

A significant decrease of CD8β+ LPL’s was observed in the group of layer-type chicken inoculated with the av2 strain compared to the non-inoculated group at day two pi (P ≤ 0.05). No differences were observed in the number of CD8β+ LPL’s in the hu1- and av2- inoculated broiler groups (Figure 22 a,b).
Figure 22: Mean number of CD8β+ lamina propria leukocytes (LPL's) per three crypts of five microscopic fields at 200 fold magnification in SPF-layer-type chicken (a) and broilers (b). Different letters indicate significant differences between groups (ANOVA, Tukey $P \leq 0.05$). $n = 5$ birds / group and day. dpi = days post inoculation. Error bars indicate the standard deviation.
5.2.3.2.3 Monocyte/macrophage- like KUL-01+

The group of hu1-inoculated SPF- layer-type chicken showed a significant decrease ($P \leq 0.05$) at day two pi compared to the control group. The macrophage-like KUL-01+ cells were significantly decreased at day three pi in av2 - inoculated broilers ($P \leq 0.05$) compared to the non-inoculated group (Figure 23 a,b).
Figure 23: Mean number of KUL-01+ cells per three crypts of five microscopic fields at 200 fold magnification in SPF-layer-type chicken (a) and broilers (b). Different letters indicate significant differences between groups (ANOVA, Tukey \( P \leq 0.05 \)). \( n = 5 \) birds / group and day. dpi = days post inoculation. Error bars indicate the standard deviation.

5.2.3.3 Cytokine expression

5.2.3.3.1 IFN-\( \gamma \) expression

Broiler chicken inoculated with the av2 strain showed a significant increase of IFN-\( \gamma \) expression at two dpi \( (P \leq 0.05) \). No differences were found between the hu1 and av2 inoculated groups of both, broilers and SPF-laying-type chicken, at any other time-point compared to the non-inoculated control groups (Figure 24 a-d).
5.2.3.3.2 IL-6 expression

The av2 strain showed an significant increase of IL-6 mRNA expression in caecum tissue of layer-type chicken at day one pi compared to the non-inoculated controls ($P \leq 0.05$). No IL-6 expression was measurable in the group of av2 - inoculated broilers at day one pi. At two dpi no IL-6 expression was measurable in neither, the group of SPF-layer-type chicken, nor in the broiler group inoculated with the av2 strain. The hu1 strain showed no significant differences compared to the non-inoculated control groups at the investigated time points (Figure 25 a-d).
Figure 25: Quantification of IL-6 mRNA expression in caecum of SPF-layer-type chicken (a, b) and broiler (c, d) infected with the respective C. jejuni strain at 1 dpi (a) and (b) and 2 dpi (c) and (d). n = 5 birds / group. Data corrected for variation by 28S mRNA levels and presented as 35-Ct in mRNA expression of Cj. - and diluent inoculated birds. Letters indicate significant differences between groups (Kruskal-Wallis-Test P ≤ 0.05). dpi = days post inoculation. Presented is the mean/ group. Error bars indicate the standard deviation.
6 Discussion

In our study we compared human and avian *Cj.* isolates for their ability to induce immune reactions in chicken. We hypothesised that strains of different origin may vary in their colonisation ability in the chicken.

6.1 Influence of the investigated *C. jejuni* strains on general health aspects of chicken

None of the investigated *Cj.* strains induced any clinical signs or pathological lesions throughout the five conducted experiments in three-week old chicken. The body weight development of both, SPF-layer-type chicken and broilers was equal to the non-inoculated control groups. However, in Experiment 5, the group of broilers inoculated with the av2 strain showed a significant lower weight gain at day one pi than the control and the hu1 inoculated group. We may speculate that the difference was by chance due to the fact, that both genders were used in this study. Being picked randomly for necropsy, the broiler group inoculated with the av2 strain may have included more females than the other two groups at one dpi. Unfortunately this was not documented. Overall, these results support the hypothesis of *Cj.* being a commensal of the chicken (WASSENAAR 2011). In contrast *Cj.* can induce clinical symptoms such as gastroenteritis and diarrhoea experimentally (RUIZ-PALACIOS et al. 1981) and even in the field in chicken. Further, due to the fact that some immunological data changes were determined in our study after *Cj.* inoculation, we cannot support the hypothesis of *Cj.* being a commensal of the chicken intestine. We may speculate that the chicken immune system might play a regulatory role on the outcome of the infection leading to a colonisation without inducing clinical symptoms or pathological lesions. We also can only speculate that other factors may contribute to *Cj.* – induced lesions and an outbreak of disease. Secondary pathogens both, enteric or systemic may pioneer the chicken intestine for *Cj.* to induce clinical and pathological symptoms. Also environmental stressors like wrong light regime and bad air quality conditions in chicken houses or high bird densities at flock level might lead to the outcome of disease. This was demonstrated for *Salmonella typhimurium* in
6.2 Colonisation properties of the investigated *C. jejuni* strains and contributing factors

6.2.1 Colonisation pattern of the investigated *C. jejuni* strains in the caecum

Our results clearly demonstrate differences between the strains in their colonisation pattern of the caecum in three-week old SPF-layer-type chicken. Hänel *et al.* (2004, 2009) investigated different *Cj.* isolates *in vitro* in primary chicken gut epithelial cells and in the human gut cell line Caco-2. They compared colonisation and invasion patterns of human, avian and bovine originated *Cj.* strains. Based on their results in chicken gut epithelial cells, they clustered the investigated strains in different categories independent of their origin: no-colonisation – weak or delayed colonisation – strong colonisation (Hänel *et al.* 2004; Hänel *et al.* 2009). Further, all investigated strains adhered to the human Caco-2-cells. Based on these results they suggest that other reservoirs, beside poultry, might play an important role in human *Campylobacter* cases (Hänel *et al.* 2009). In our study there were no clear consistent differences between human and avian isolates with regard to their colonisation pattern. Both, human and avian isolates were re-isolated from the caecum at equivalent bacterial counts per gram caecal content.

The two human reference strains NCTC11168 (hu3) and 81-176 (hu2) have been often used in *Campylobacter* research in both, mice and chicken (Jones *et al.* 2004; Bereswill *et al.* 2011). Interestingly, in our study hu2 was first re-isolated at day seven pi at a low level (10⁴ CFU) in the caecal content of only one bird out of six. The detection rate increased to 10⁸ CFU with a ratio of 100% positive chicken at the end of the experiment (21dpi). The hu2 has been described to be a good colonizer of the chicken caecum with 100% positive birds within the first week after inoculation if an inoculation dose of 10⁷ CFU was administered orally (Jones *et al.* 2004). However, in the study of Jones *et al.* (2004) two-week old chicken were inoculated with a
higher dose than in our study (JONES et al. 2004). This might explain the delayed colonisation in our study and also gives evidence of a dose dependency. Further, JONES et al. (2004) used younger chicken in their study than we did (JONES et al. 2004). This suggests that the age of birds at inoculation seems to play a role in colonisation. The human reference strain NCTC11168 (hu3) was previously shown to be a bad or non-coloniser of one day old and two-weeks-old SPF chicken. Successful colonisation was only observed after oral inoculation with a dose of $10^9$ CFU in one-day-old birds (AHMED et al. 2002; JONES et al. 2004). In our study hu3 showed a detection rate of $10^7$ CFU per gram caecal content after oral inoculation of three-week-old SPF-layer-type chicken at all investigated time points. At three days post inoculation only 50% of the birds were positive for *Campylobacter* but after seven dpi all birds were tested positive for *Cj*. This observation also indicates that older birds seem to be more susceptible for colonisation after inoculation with the hu3 strain and therefore also point to an age dependency for *Cj*. colonisation.

The avian strain av518 (av3), which originated from an organic turkey hen was first detectable at day 14 pi in the caecal content after inoculation of three-week-old SPF-layer-type chicken. Compared to the delayed onset of the av3 strain in SPF-layer-type chicken in our experiments, 44-day-old turkey hens had been also inoculated with the av3 strain with $10^4$ CFU. This resulted in all birds being positive at day five pi after inoculation (Glünder unpublished data). This unpublished data also suggests an influence of the species genetic background of the host for successful colonisation. The influence of the genetic background of the host is known from other avian pathogens such as Infectious bursal disease virus (IBDV), Marek’s disease or *Salmonella* enteritidis (KAISER u. LAMONT 2001; SARSON et al. 2008; ABASHT et al. 2009; ARICIBASI et al. 2010). Gene families encoding for immunoglobulins, MHC molecules and cytolytic proteins seem to play a crucial role for a higher host resistance towards a pathogen (SARSON et al. 2008). For IBDV, SPF-layer-type-chicken, seem to be more susceptible than commercial broilers. SPF-chicken showed more severe clinical signs, mortality and higher fold change in circulating cytokines than commercial broilers (ARICIBASI et al. 2010). Further evidence for a host genetic influence is given by the results from Experiment 5. Av2 inoculated
broilers in Experiment 5 showed a higher and earlier re-isolation rate than the SPF birds. This also might indicate a higher susceptibility of broilers for this *Cj*. strain in broilers than SPF-layer-type chicken. The human *Cj*. isolate B2 (hu1) has been characterised as highly virulent for human (TAREEN et al. 2010). Interestingly the hu1 strain could not be re-isolated from the caecum of SPF-layer-type chicken between one and 21dpi in either experiment. In contrast, Experiment 5 revealed that commercial broilers were more susceptible for the hu1 strain also suggesting a genetic influence on *Campylobacter* colonisation. This can be based on microbiome differences. It was shown that the host genetic background influences the microbiome. Even the gender seems to play a role in the microbiome development (ZHAO et al. 2013). It can also be suggested that the different composition of layer and broiler feed may have contributed to the differences in colonisation. Broiler feed harbours a higher amount of crude protein which, may lead to a different gut flora development and therefore modify the *Cj*. colonisation. It was shown that a change in diet can lead to a change in microflora composition (HAMMONS et al. 2010). In broilers it was shown that changes in cereal influence *Salmonella* enteritidis colonisation and even gut wall morphology (TEIRLYNCK et al. 2009a; TEIRLYNCK et al. 2009b).

Gut flora composition was also shown to have an effect in mice inoculated with *Cj*. The gut flora composition of wild-type and gnotobiotic mice holding a mouse-flora composition seem to act as a natural barrier against colonisation of *Cj*. In contrast, it was possible to colonise germ-free mice successfully with the same *Cj*. strain when being reconstituted with human intestinal flora (BERESWILL et al. 2011). Several authors demonstrated in two-day- and four-day-old broilers that the use of antimicrobials leads to higher Salmonella counts in the gut by disrupting the normal intestinal flora (RANTALA u. NURMI 1974; SMITH u. TUCKER 1978). This enhancing effect on colonisation may also be suggested for *Campylobacter* and has to be investigated further.

Overall our results suggest that colonisation of the chicken intestinal tract is not only dependent on the colonising strain but also on the age and dose of inoculation. Further, *Cj*. colonisation is possibly influenced by the host’s genetic background.
ZAUTNER et al. (2012) also suggest a differential host prevalence for *Campylobacter* isolates of different origin based on differences in their genetic markers (ZAUTNER et al. 2012). However, it is known that genomic changes occur in *Campylobacter* after passage in the chicken gut (HÄNEL et al. 2009). Nevertheless, our study gives evidence that strain variation is not essentially correlated with the origin, either human or avian, and the investigated host.

### 6.2.2 Colonisation pattern of the investigated C. jejuni strains in the liver

Still being discussed to contribute to avian hepatitis (JENNINGS et al. 2011) our results identified only one strain invading the liver after infection of clinically healthy birds. Only the av2 strain, which was isolated from the liver of a laying hen, was re-isolated from the liver of av2 inoculated SPF-layer-type-chicken (Exp. 3 and 5) and broilers (Exp. 5) in a comparable way. These findings clearly suggest that *Campylobacter jejuni* may overcome the intestinal barrier, invade the intestinal tract and become systemic. Earlier isolations of *Cj* from different organs like spleen, heart blood, gall bladder suggest the possibility of a systemic appearance (SANYAL et al. 1984), however the factors contributing to a systemic campylobacteriosis are still unclear. Secondary pathogens may contribute to a systemic form and thereby may result in the colonisation of other organs. It is known that IBDV enhances *Cj* colonisation in chicken (SUBLER et al. 2006) also providing evidence for an important role of host’s immune status for control of *Campylobacter* colonisation. The mechanisms that lead to a systemic spread of *Cj* have to be elucidated further.

Other authors have managed to re-isolate *Cj* from livers of chicken after oral inoculation. However, no correlation between liver invasion and the caecal bacterial load was determined (HÄNEL et al. 2004; HÄNEL et al. 2009). As in colonisation of the chicken intestine, the infectious dose may play a role in liver colonization. HÄNEL et al. (2009) used doses of $10^8$ CFU for their infection study with human and bovine isolates. They were able to re-isolate several isolates from the liver. Unfortunately, they do not give any information about which strain was re-isolated from the liver (HÄNEL et al. 2009). In our study we used the initial dose of
$10^4$ CFU resulting in only one strain invading the liver indicating, beside other contributing factors, a strain dependency.

**6.3 Interaction of the immune system with *C. jejuni***

**6.3.1 Humoral immune response**

Maternal antibodies are suggested to protect chicken within the first three weeks of age against *Campylobacter* colonisation and infection. In contrast, experimental studies but also observations in the field indicate that even day-old-maternally antibody-positive chicken can be colonised successfully with *Cj*. (CAWTHRAW u. NEWELL 2010).

No induction of *Cj*.-specific IgM-type serum antibodies in none of the inoculated groups was detected.

Of the four tested human isolates only the hu3 strain induced *Cj*.-specific IgG-type serum antibody levels at 14 and 21 days pi in SPF-layer-type chicken in comparison to the other strains. However, no consecutive reduction of the *Cj*. bacterial count in the caecum was observed.

The strain hu1 was not re-isolated from the caecum and therefore no detectable antibodies may have been induced. The hu2 strain showed a delayed colonization, which might explain a lack of serum antibodies at already three weeks pi. On the other hand, the av3 strain also showed a delayed colonisation (14dpi). In this case serum antibodies were detected above the cut-off value at 21 dpi. Other factors seem to contribute to the induction of humoral immunity. Surprisingly the hu4 strain, which showed high colonisation rates in the caecum, also did not induce a humoral immune response. Being a non-toxin producer, this strain may not be immunogenic enough to induce serum antibodies.

Interestingly, all avian strains tested induced *Cj*.-specific IgG antibodies at day 21 pi (Fig. 9). The strain av1 already had induced serum IgG-type antibody levels above cut-off from day seven onwards till 21 dpi. But, there was no influence on the caecal *Cj*. count in any of the av strains – inoculated groups. Nevertheless, avian isolates seem to be more immunogenic for chicken than isolates of human origin. However,
the humoral response seems to be induced at a later time-point after inoculation. Our experiments were terminated three weeks pi. A longer lasting experiment may be necessary to detect possible effects of antibodies on bacterial counts. Overall, the detected OD values were at low levels. This might be due to the fact that protein as a source for antigen was used in the ELISA instead of attenuated Campylobacter (HAAS et al. 1999).

The fact that Cj. is located in the mucus within the crypts (BEERY et al. 1988) can be responsible for a bad stimulation of circulating serum antibodies. Local antibodies, mainly IgA, from gut washings have to be investigated (LEBACQ-VERHEYDEN et al. 1974).

6.3.2 Cell-mediated immune response

There is still a lack of knowledge with regard to the cell-mediated immune response in chicken after challenge with Cj. Studies mainly focused on the expression of different cytokines after Cj. challenge. Overall, there were only little changes in T cell numbers throughout the experiments. But, some effects were observed on the level of IFN-γ and IL-6 expression in caecum samples. Two avian strains showed an up-regulation in cytokine expression at day three pi. After inoculation of av1 both, IFN-γ and IL-6 was significantly increased and av3 inoculated SPF-layer-type chicken showed a significant increase of IL-6 expression at day three pi compared to the control group. On the other hand the hu4 strain showed a significant decrease of IFN-γ expression compared to the non-inoculated controls. Further no IL-6 expression was detectable at day three pi. Also the hu3 strain showed no measurable Ct values for both, IFN-γ and IL-6 expression in caecum samples, which can be indicative for a down regulation of these cytokines. Overall, our data gives some evidence for a down-regulation of the immune system resulting in a possible immunosuppression at the early stage of colonisation allowing Cj. further colonisation. It may be speculated that down-regulation of cytokines and subsequent lack of T cell response during the first days after inoculation may lead to an insufficient humoral immune response. This may be supported by the ELISA
results of the avian strains, with all three strains inducing IgG-type serum antibodies not earlier than seven days pi.
Beside our results, there is more evidence that the immune response within the first days post inoculation may play a crucial role for the outcome of *Cj.* colonisation and infection in chicken (SMITH et al. 2008; MEADE et al. 2009; SHAUGHNESSY et al. 2009). To elucidate this further Experiment 5 was performed, not only to get a view at the earlier time points of the immune response, but also to confirm our prior results. Consequently, in Experiment 5 we focused on earlier time points after *Cj.* inoculation conducting the first necropsy at day one pi and selected two strains (hu1 and av3) from the prior experiments.

The assumption that the immune response occurs at the early stage of colonisation was confirmed. On the one hand a significant increase of CD4+ and CD8β+ LPL’s at day one pi was observed in the hu1 inoculated group of SPF-layer-type chicken compared to the control birds. Additionally, av2 inoculated SPF-layer-type chicken showed a significant higher number of TCRγδ+ IEL’s as well as a significant increase of IL-6 at day one pi. Contrariwise, the group of av2 inoculated SPF-layer-type chicken had a significant lower number of CD8β+ LPL’s as well as non-measurable IL-6 expression compared to the non-inoculated group on day two pi. This may be indicative for an immunosuppressive role of *Cj.* in chicken during the process of colonisation. Broilers inoculated with the hu1 strain showed a higher number of CD4+ LPL’s at day one also indicative for an immune response prior to day three pi. Av2 inoculated broilers revealed a significant reduction in the percentage of CD4+ IEL’s at one and two days pi. Additionally, at day one pi a higher number of CD4+ LPL’s was observed compared to the non-inoculated group. This might be indicative for a migration of CD4+ T cells from the intraepithelial border to the lamina propria and therefore the crypt area, where *Cj.* is mainly located.

Surprisingly, other studies observed significant differences on IFN-γ or IL-6 mRNA level mainly in avian cell lines. However, due to performing their research in vitro some influencing factors such as environmental factors or feed composition, which may influence the hosts immune response, may be missing. Additionally, their investigation took place during the first few hours pi (SMITH et al. 2008).
Consequently, an *in vivo* investigation during the first hours after *Cj.* inoculation may shed more light in the host immune response.

### 6.4 Conclusion and further perspectives

Nowadays, some authors define *Cj.* to be a commensal of the chicken intestinal tract (LEE u. NEWELL 2006). Further it is stated, that *Cj.* is part of normal intestinal flora and humans are supposed to be collateral host rather than direct targets of the bacteria (WASSENAAR 2011). However, clinical signs and lesions can be observed in *Campylobacter* positive chicken and also can be induced experimentally. These findings are directly associated with *Cj.* although secondary pathogens have to be regarded as supporters or to evocate the outcome of *Campylobacter* infection. Stress, provoked by high bird density in the flock or wrong light regime may also contribute to *Cj.* infection and lead to a clinical disease.

Our results indicate a host-bacterial interaction. Further, our study, as well as other working groups showed that the chicken, as a host, deals with *Cj.* in an immunological way. It seems that immune responses directed towards *Cj.* appear early. This was shown *in vitro* as well as *in vivo* studies by other authors (SMITH et al. 2005; SMITH et al. 2008; MEADE et al. 2009). In contrast, our results of the immunological investigation give some evidence of a possible *Cj.* induced immune suppression supporting the colonisation process in the caecum. A temporarily down-regulation of cytokines may be a result of a short-time persistence of *Cj.* in the epithelium (VAN DEUN et al. 2008)

We could show in our study that many factors contribute to caecum colonisation of *Cj.* isolates of avian and human origin. On the one hand, the dose of inoculation seems to play an important role for colonisation of the chicken caecum. Further, the genetic background seems to play a crucial role with broilers being more susceptible than SPF-layer-type chicken. Further studies with other lines have to be performed to get a deeper insight. But we have only investigated a small number of *Cj.* isolates and more work has to be done to find out more about the host – *Campylobacter* interaction. Further, the *Cj.* strain itself influences the colonisation of the chicken gut.
We could clearly show differences in the colonisation pattern, however, it did not correlate with the origin of the isolate.

Age is also a factor, which may contribute to the colonisation of the chicken gut, which has to be investigated further. The chicken used in our study were all inoculated at the age of three weeks. However, HÄNEL et al. (2004) used chicken at the age of nine days post hatch resulting in both higher bacterial counts in the caecum and more strains being detected in the liver (HÄNEL et al. 2004). But, beside the different age, they also used a higher initial dose for their study. All these mentioned factors making comparisons between each study very difficult and further work has to be done to get more information about the bacterium-host-interaction.

The disturbance of the gut microbiome may also influence Cj. colonisation and infection. It is known that Lactobacillus ssp. counteracts with Cj. and leads to a lower bacterial burden in the gut. Nevertheless, no complete clearance was obtained (STERN et al. 2006). Due to our result, broilers seem to be more susceptible than SPF-layer-type chicken, which might also be influenced by the intestinal flora. Broiler feed holds more crucial protein, which might enhance or support Cj. Investigation of the microbiome in correlation with Cj. may give a deeper insight in bacterial interactions in the chicken gut.
7 Summary
Colin Pielsticker

**Investigations on the humoral and cell-mediated immune response in chicken after inoculation with *Campylobacter jejuni* strains of human and avian origin**

Poultry and especially chicken and raw chicken meat are the major source for *Cj*. induced acute gastro-enteritis in humans in industrialised countries. Nowadays *Cj.* is the most common bacterial food-borne pathogen. Despite regarded as a commensal of the chicken intestinal tract *Cj.* is known to cause symptoms and pathological lesions in chicken.

The factors contributing to colonisation of both, human and chicken are still not completely understood. Additionally there is a lack of knowledge regarding the immune mechanisms involved in *Cj.*-chicken-interaction.

In our study we have investigated different *Cj.* isolates of human and avian origin. In the first four conducted experiments we determined the colonisation pattern of these different isolates in the caecum as well as in the liver. Further, we investigated humoral and cellular immune parameters to get an insight in the host-bacterial-interaction. These experiments revealed some interesting differences with regard to colonisation of caecum and liver. One out of seven strains was identified to invade the liver, whereas one human strain was not able to colonise the caecum of SPF-layer-type chicken. On the other hand, the origin of the isolate seems to play a minor role in colonisation, with three of four human strains colonising the chicken gut. The investigation of the T cell and innate immune response revealed only minor differences between *Cj.* – and diluent inoculated chicken. However, our results are indicative for an immune response prior to three days pi. The results of the antibody ELISA showed that all avian strains induced IgG-type antibodies. On the other hand, only one human strain showed levels of IgG-type antibodies above cut-off.

Based on the results of the first four experiments, the fifth experiment focused on two major aspects. On the one hand side, we wanted to investigate the immune response prior to three day pi. On the other side we wanted to determine if there may be any
possible differences between the typical experimental SPF-layer-type chicken and commercial broilers after inoculation with *Cj*. For that we inoculated SPF-layer-type chicken and commercial Ross broilers with two *Cj*. isolates already used in the first conducted experiments. Again we focused on colonisation pattern as well as on the T cell and innate immune parameters. As a major modification to the other experiments we determined all parameters at earlier time-points. The fifth experiment not only confirmed results from the first experiments but also our suggestions. As in Experiment 1, SPF-layer-type chicken were not colonised by the hu1 strain, whereas the same strain was re-isolated from caecal content of broilers giving evidence for broilers to be more susceptible than SPF-layer-type chicken. Further, the av2 strain invaded the liver of both, SPF-layer-type chicken and broilers as in Experiment 3. Our suggestion, the immune system responding during the early phase of colonisation, was confirmed with more significant differences on the level of T cells as well as on the level of cytokine expression at days one and two pi. Overall, on the one hand our results first seemed to support the hypothesis of *Cj.* being a commensal of the chicken gut without inducing any clinical signs or pathological lesions in none of our conducted experiments. However, this hypothesis has to be seen from different angles. We demonstrated experimentally that the chicken immune system interacts with *Cj.* at the early stage of colonisation. T cell and innate immune response seems to play a role during the early phase, whereas the induced humoral immune response seems to take part at a later time-point after inoculation.
8 Zusammenfassung

Colin Pielsticker

Untersuchungen zur humoralen und zell-vermittelten Immunantwort in Hühnern nach Inokulation mit humanen und aviären Campylobacter jejuni Stämmen


Sowohl für den Menschen als auch für das Huhn sind die verantwortlichen Kolonisationsfaktoren noch nicht umfassend bekannt. Weiterhin gibt es auch nur sehr wenige Informationen hinsichtlich der Immunreaktionen im Huhn nach einer Infektion mit Cj.


Abschließend ist anzumerken, dass unsere Ergebnisse der Mikrobiologie als auch der klinischen, pathologisch-anatomischen und histologischen Untersuchungen zwar die These eines Kommensalen unterstützen. Allerdings konnten wir auch nachweisen, dass sich das Huhn als Wirt immunologisch mit Cj. auseinandersetzt, welches im Widerspruch zu einem reinen Kommensalen steht. Weiterhin scheint das angeborene Immunsystem sowie die T-zell-vermittelte Immunantwort während der frühen Phase der Besiedlung eine Rolle zu spielen. Im Gegensatz dazu scheint die humorale Immunantwort zu einem späteren Zeitpunkt nach Inokulation zu agieren.
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10 Appendix

10.1 Animals

10.1.1 SPF layer chicken
(Valo® Lohmann-Tierzucht, GmbH & Co, Cuxhaven, Germany)

SPF chicken are free from:
Avian Adeno Viruses, group1; Avian Encephalomyelitis Virus; Avian Infectious Bronchitis Virus; Avian Infectious Laryngotracheitis Virus; Avian Leukosis Viruses/RSV; Avian Nephritis Virus; Avian Orthoreoviruses; Avian Reticuloendotheliosis Virus; Chicken Anemia Virus; Egg Drop Syndrome Virus; Fowlpox Virus; Infectious Bursal Disease Virus, Serotype 1 and Serotype 2; Influenza A Virus; Marek’s Disease Virus; Mycobacterium avium; Mycoplasma gallisepticum; Mycoplasma synoviae; Newcastle Disease Virus; Salmonella pullorum; Salmonella ssp.; Turkey Rhinotracheitis Virus

10.1.2 Broilers
(BWE-Brüterei Weser-Ems GmbH & Co. KG, Visbek, Rechterfeld, Germany)
Ross #538 ; non-vaccinated at hatchery

10.2 Food composition
Ingredients from manufacturer’s specifications

10.2.1 Layers
Complete feeding stuff for layers; Legemehl (Deuka, All-mash L)
crude protein 16.5%
 crude fat 4.2%
crude ash 12.6%
crude fiber 3.1%
10.2.2 Broilers

The broilers received food for their correspondent age. The sequence was as follows:

**Landkornstarter** (Deuka)
crude protein 21.5%
crude fat 4.9%
crude ash 3.4%
crude fiber 5.6%
100 mg Monensin-Natrium (coccidiostatic)
up to 15 days post hatch

**Landkornmast** (Deuka)
crude protein 21.0%
crude fat 5.7%
crude ash 3.5%
crude fiber 5.1%
100 mg Monensin-Natrium (coccidiostatic)
up to 20 days post hatch

**Landkornendmast** (Deuka)
crude protein 20.0%
crude fat 6.6%
crude ash 3.6%
crude fiber 4.8%
without coccidiostatic
10.3 Composition and preparation of the used reagents

**Phospahte Buffered Saline** (PBS-M 10x); pH 7.4; 10 times concentrated

- KH$_2$PO$_4$ 2 g Merck, Darmstadt, Germany
- NA$_2$HPO$_4$ 29 g Sigma, Steinheim, Germany
- NaCl 80 g Sigma, Steinheim, Germany
- KCl 20 g Sigma, Steinheim, Germany

Ad 1000 ml Aqua Bidest, pH 9.6

For use it has to be diluted 1:10 with Aqua Bidest to receive PBS-M 1x.

**Buffered Formalin**; pH 7

- Formaldehyde solution, non-acidic 100 ml Roth GmbH, Karlsruhe, Germany
- NA$_2$HPO$_4$ 4 g Sigma, Steinheim, Germany
- NAH$_2$PO$_4$ * H$_2$O 6.5 g Sigma, St. Louis, USA

Ad 900 ml Aqua Bidest, pH 9.6

**FACS buffer**

- Bovine serum albumin 10 g PAA Laboratories Company, Pasching, Austria (pH 7.0)

Ad 1000 ml of PBS-M 1x

**Standard – I – nutrient broth**

(Merck, Darmstadt, Germany); pH 7.5

Ad 25 g in 1000 ml of aqua dest while heating in a boiling water bath and autoclave after preparation

**Coating – puffer**

- Na$_2$CO$_3$ 1.59 g Sigma, Steinheim, Germany
- NaHCO$_3$ 2.90 g Sigma, Steinheim, Germany
- NaN$_3$ 0.20 g Sigma, Steinheim, Germany

Ad 1000 ml Aqua Bidest, pH 9.6
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