Evaluation of intervention measures to control *Campylobacter jejuni* in broiler chickens

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SAMENVATTING

CURRICULUM VITAE

DANKWOORD
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees celsius</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>ABC</td>
<td>ammonium bicarbonate</td>
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<tr>
<td>ACN</td>
<td>acetonitril</td>
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<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>BB</td>
<td>blocking buffer</td>
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<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
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<tr>
<td>C. jejuni</td>
<td>Campylobacter jejuni</td>
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<tr>
<td>cCIN</td>
<td>micro-encapsulated trans-cinnamaldehyde</td>
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<td>cfu</td>
<td>colony-forming units</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EB</td>
<td>extraction buffer</td>
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<td>EC</td>
<td>Ethical Committee</td>
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<td>EGCG</td>
<td>epigallocatechin gallate</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ESI-QUAD-TOF</td>
<td>electrospray ionization quadrupole time of flight</td>
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<td>EU</td>
<td>European Union</td>
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<tr>
<td>FCA</td>
<td>Freund’s complete adjuvants</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FIA</td>
<td>Freund’s incomplete adjuvants</td>
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<td>FoodNet</td>
<td>Foodborne Diseases Active Surveillance Network</td>
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<td>GBS</td>
<td>Guillain-Baré syndrome</td>
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<tr>
<td>GI</td>
<td>gastro-intestinal</td>
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<tr>
<td>GRAS</td>
<td>generally recognized as safe</td>
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<td>h</td>
<td>hour</td>
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<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<td>HCl</td>
<td>hydrochloric acid</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>IgY</td>
<td>immunoglobulin Y</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>log</td>
<td>logarithm</td>
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<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>Mbp</td>
<td>megabasepairs</td>
</tr>
<tr>
<td>mCCDA</td>
<td>modified charcoal cefoperazone deoxycholate agar</td>
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<tr>
<td>MCFA</td>
<td>medium-chain fatty acids</td>
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<tr>
<td>MCP</td>
<td>methyl-accepting chemotaxis protein</td>
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<td>MHB</td>
<td>Mueller-Hinton broth</td>
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<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
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<td>min</td>
<td>minute</td>
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<td>mL</td>
<td>milliliter</td>
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<td>mM</td>
<td>millimolar</td>
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<tr>
<td>MOMP</td>
<td>major outer-membrane protein</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>NB2</td>
<td>Nutrient Broth No.2</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>$P$</td>
<td>p-value</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PDAC</td>
<td>plant-derived antimicrobial compound</td>
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<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
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<td>pi</td>
<td>post-inoculation</td>
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<td>ppm</td>
<td>parts per million</td>
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<tr>
<td>RAPD</td>
<td>random amplification of polymorphic DNA</td>
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<td>rpm</td>
<td>rounds per minute</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>SACS</td>
<td>surface-accessible carbohydrate structure</td>
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<td>SCFA</td>
<td>short-chain fatty acids</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>spp</td>
<td>species</td>
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<tr>
<td>TAT</td>
<td>twin-arginine translocase</td>
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<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
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<td>US</td>
<td>United States</td>
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<td>V</td>
<td>volt</td>
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<td>vol</td>
<td>volume</td>
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<tr>
<td>WB</td>
<td>washing buffer/western blot</td>
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<td>wt</td>
<td>weight</td>
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CHAPTER 1: LITERATURE REVIEW

Adapted from:
CHAPTER 1: LITERATURE REVIEW

1.1 POULTRY AS A HOST FOR ZOONOTIC CAMPYLOBACTER SPP.

1.1.1 General introduction

*Campylobacter* species are Gram-negative bacteria that belong to the family of the *Campylobacteraceae*. These micro-organisms generally possess a single polar flagellum at one or both sides of their cell end, promoting motility. Campylobacters are unable to ferment or oxidize carbohydrates and therefore depend on amino acids and Krebs cycle intermediates as primary energy sources. The bacteria are microaerophilic, only growing at reduced oxygen levels, and have a minimal growth temperature of over 30°C. Both these growth requirements make it unlikely for campylobacters to multiply in the outside environment (Snelling *et al.* 2005). Instead, they seem to be highly adapted to some warm-blooded animal reservoirs, in which they primarily occur as commensals.

In humans, campylobacters are now the leading cause of bacterial gastro-enteritis in many developed countries. Campylobacters generally do not cause large outbreaks of gastro-intestinal illness since over 90% of campylobacteriosis cases in humans are sporadic (Snelling *et al.* 2005). Most *Campylobacter* infections are self-limiting and thus do not require antimicrobial therapy (Butzler, 2004). These infections generally last for up to seven days and are accompanied with extremely diverse clinical manifestations, ranging from a complete absence of symptoms to fulminating sepsis. In a small number of cases, however, the infection may develop into long-term complications and even death. These post-infectious complications include arthritis, Reiter syndrome and Guillain-Barré syndrome (GBS), an acute auto-immune disease affecting the peripheral nervous system leading to muscle weakness and sometimes paralysis, and that can even be life-threatening (Butzler, 2004; Snelling *et al.* 2005). Although during recent years the number of registered campylobacteriosis cases has declined slightly in some parts of the world outside the EU (Ailes *et al.*, 2008), the overall disease burden is still noteworthy and registered cases in the EU are steadily increasing (EFSA, 2012). The true incidence of campylobacteriosis in industrialized countries is uncertain since many unreported infections occur for every diagnosed case (Havelaar *et al.*, 2012). Within the European Union, campylobacteriosis has been the most frequently reported zoonotic disease in humans as from 2005. In 2010, the overall EU notification rate was 48.6 cases per 100,000 inhabitants (EFSA, 2012). Thanks to
a significant decrease in campylobacteriosis cases compared to previous years (2005 - 2009), Belgium remained below this average with a notification rate of 28.0 per 100,000 inhabitants in 2010 (EFSA, 20011a; 2012). In the United States, the Foodborne Diseases Active Surveillance Network (FoodNet) reported an incidence of culture-confirmed Campylobacter infections in the FoodNet sites of 12.7 per 100,000 persons in 2006 (Ailes et al., 2008). These numbers represent a 30% decline compared to the 1996 situation, but the incidence still remains above the national health objective. Most other regions of the world report a higher disease incidence, with strikingly high numbers in New Zealand in 2003 of almost 400 cases per 100,000 people (Baker et al., 2007). One group of campylobacters, the “thermotolerant” or “thermophilic” Campylobacter species (containing C. jejuni, C. coli, C. lari, C. upsaliensis and C. helveticus and for simplicity hereafter referred to as Campylobacter) are especially associated with human gastro-intestinal disease (Snelling et al., 2005). Campylobacter enteritis in humans is, however, mainly caused by C. jejuni (EFSA, 2012). In 2010, 35.7% of the isolates were identified as C. jejuni, representing 93.4% of the cases characterized at species level. C. coli, C. lari and C. upsaliensis accounted only for respectively 2.3%, 0.22% and 0.006% of the isolates. The remainder of the isolates included other species or were not speciated or not known. C. jejuni is able to infect humans at doses as low as 500 colony-forming units (Snelling et al. 2005).

1.1.2 Campylobacter prevalence in animals and the environment

Paradoxically, despite their fastidious, fragile nature, campylobacters are highly prevalent in the environment and can survive for prolonged periods both inside and outside a suitable host (Newell, 2002; Murphy et al., 2006). How the microorganism copes with stresses encountered in the environment still remains enigmatic but clearly, campylobacters developed some survival mechanisms to overcome these stressors (Murphy et al., 2006). The presence of highly mutable sites in the C. jejuni genome (see 1.2.1) is responsible for its rapid adaptation
in a novel host (Jerome et al., 2011). Campylobacters can be frequently found in surface water and is part of the natural intestinal microbiota of a wide range of wild and domestic animals, especially poultry (Newell, 2002, Whyte et al., 2004; Abulreesh et al., 2006; Young et al., 2007; Ogden et al., 2009, Jokinen et al., 2011). The estimated Campylobacter prevalence in poultry and non-poultry farm animals depends on season, age of animal, flock or herd size and type, diet, husbandry practices and geography, with overall C. jejuni being the most isolated species (Kuana et al., 2008; McDowell et al., 2008; Zweifel et al., 2008; Ellis-Iversen et al., 2009; Messens et al., 2009; Näther et al., 2009; EFSA, 2010b; EFSA, 2012; Jorgenson et al., 2011).

1.1.3 Poultry as a natural host for zoonotic Campylobacter

Chickens are a natural host for Campylobacter species (EFSA, 2012). The probability of a flock to become colonized increases during rearing, resulting in on average 60% to 80% of the analyzed broiler flocks to be positive for Campylobacter species in general at slaughter age worldwide (Herman et al., 2003; Rasschaert et al., 2006; Kuana et al., 2008; Reich et al., 2008; EFSA, 2010b). Striking differences in EU prevalence do exist. In 2008, almost all broiler batches in some Northern-European countries like Estonia, Norway and Finland were free of Campylobacter, while a strikingly high prevalence of 100% was reported for Luxembourg. With a prevalence of 31.0% Campylobacter-colonized broiler batches in 2008, Belgium stayed below the average EU prevalence of 71.2%. In general, C. jejuni was found to be the dominating species in broilers, although in some Southern European Member States, especially Malta and Luxembourg, C. coli was the most commonly isolated Campylobacter species (EFSA, 2010c). Colonized poultry flocks might contaminate the surrounding environment by which Campylobacter is able to spread further and contaminate other farms or humans (Jonsson et al., 2010). Since C. jejuni is the most important species causing disease in humans, the focus of this thesis will be primarily on this species.

1.1.4 Colonization of broiler chickens with C. jejuni at the farm

From day-of-hatch until the broiler chickens are transported to the abattoir, the animals can encounter several risk factors contributing to their colonization with C. jejuni. As a consequence, the Campylobacter epidemiology in broiler flocks is quite complex. An overview of the risk factors involved in the environmental transmission of Campylobacter to broiler flocks is given in table 1.
1.1.4.1 *Initial broiler flock colonization*

Initial colonization of broiler chickens probably occurs through horizontal transmission from the environment, while vertical transmission from breeder hens or carryover of infection from a positive flock to a new flock in the same house, after cleansing and disinfection, are considered to be unlikely (van de Giessen *et al*., 1992; Jacobs-Reitsma *et al*., 1995; Bull *et al*., 2006; Patriarchi *et al*., 2011). Indeed, carryover of *C. jejuni* subtypes between broiler flocks in the same house seems to occur only rarely (Barrios *et al*., 2006; Colles *et al*., 2008; McDowell *et al*., 2008). Persistent clones in the outside environment can, however, be responsible for repeated infection of multiple broiler flock rotations (Petersen & Wedderkopp, 2001; Wedderkopp *et al*., 2003; Kudirkienė *et al*., 2010).

Colonization of broiler chickens with *C. jejuni* is influenced by many factors including source of the microorganism, the infecting dose and age of the animal (Stas *et al*., 1999). Most broiler flocks become colonized only at an age of two to four weeks, after which the infection rapidly spreads to all birds, which remain colonized until slaughter (Jacobs-Reitsma *et al*., 1995; Stern *et al*., 2001b; Stern, 2008; van Gerwe *et al*., 2009). The likelihood of a flock to become colonized with *Campylobacter* increases with higher slaughter age (Berndtson *et al*., 1996b; Barrios *et al*., 2006). Also the time of year clearly has an influence. In Germany and the UK, the risk for broilers to become colonized with *Campylobacter* is highest during the summer months (McDowell *et al*., 2008; Ellis-Iversen *et al*., 2009; EFSA, 2010d; Ellerbroek *et al*., 2010; Jørgenson *et al*., 2011) and a coincident seasonality of infections in chickens and humans has been shown (Meldrum *et al*., 2005). A significant relationship was observed between several climatic factors (such as environmental temperature and amount of sunshine and rainfall) and *Campylobacter* prevalence in UK broiler flocks prior to first partial or full depopulation (Jørgenson *et al*., 2011), which could explain this observed seasonality although other factors, such as husbandry practices and biosecurity, may also be important (Allen *et al*., 2011). Finally, an increased flock size was associated with a higher probability of that flock to be colonized with *Campylobacter* (Berndtson *et al*., 1996b; Barrios *et al*., 2006).

1.1.4.2 *Sources for horizontal Campylobacter transmission to broiler flocks*

Colonized livestock and free-living animals are an important risk factor for transmitting *C. jejuni* to broiler flocks as *C. jejuni* genotypes from cattle and laying hens, present at poultry farms, can also be found in the broiler flocks (van de Giessen *et al*., 1996; Ridley *et al*., 2008a; Zweifel *et al*., 2008; Ellis-Iversen *et al*., 2009; Allen *et al*., 2011), while pigs are a source for *C. coli* (Zweifel *et al*., 2008). Bovine *Campylobacter* isolates are indeed able to
efficiently colonize chickens (Hanel et al., 2009) and might thus be a source for broiler chicken infection, although they differ significantly from chicken and human isolates by a reduced prevalence of two genetic markers especially, the \textit{dmsA} and \(\gamma\)-glutamate transpeptidase (\textit{ggt}) gene (see 1.2.2.10), important for persistent colonization of \textit{C. jejuni} in chickens (Barnes et al., 2007; Gonzalez et al., 2009). Also several other studies revealed the presence of identical \textit{C. jejuni} clones in bovines, chickens and humans (Nielsen et al., 1997; On et al., 1998; Gilpin et al., 2008; Ragimbeau et al., 2008; Hakkinen et al., 2009; Huang et al., 2009). Recently, it was shown by molecular typing that \textit{Campylobacter} strains from a broiler house and from an adjacent dairy farm were similar to those subsequently detected in the flock, indicating the importance of horizontal transmission and the risk of transmission of \textit{Campylobacter} on multispecies farms (Ridley et al., 2011). This study also indicated that bovine fecal \textit{Campylobacter} strains can colonize chickens, which was confirmed later on by Patriarchi et al. (2011). On a German farm, indistinguishable isolates were found in different flocks during the same rearing period (Ellerbroek et al., 2010). This suggests that \textit{Campylobacter} strains might be transmitted from one broiler flock to another or might point toward a common external source infecting multiple broiler flocks at the same farm.

Also rodents, flies and their larvae are potential vectors for \textit{C. jejuni} transmission to broiler flocks (Berndtson et al., 1996a; Hald et al., 2004; Nichols, 2005; Hald et al., 2008; Hazeleger et al., 2008). An association has been shown between the presence of rodents on farms and an increased risk for flocks to become colonized with \textit{Campylobacter} (McDowell et al., 2008). Another important source of infection is contaminated surface water as genotypes found in broilers can sometimes be detected in water puddles and ditch water as well, before the flocks are colonized (Bull et al., 2006; Messens et al., 2009). \textit{C. jejuni} survival in water is promoted by several factors, including biofilm formation and possibly the viable but non-culturable state, in which \textit{C. jejuni} enters when outside a suitable host (Sparks, 2009).

Personnel and farm equipment such as trucks, forklifts, pallets, crates and footwear have also been identified as potential sources of \textit{C. jejuni} infection of broilers (Ramabu et al., 2004). Also, broiler flocks on farms with three or more broiler houses, low frequency of footbath disinfectant change and decreased cleanliness of the broiler house ante-room, have an increased risk to become colonized (McDowell et al., 2008).

Finally, partial depopulation/thinning of broiler flocks (early removal of a part of the birds) has been implicated as a potential risk factor for \textit{Campylobacter} colonization of the remainder of the animals of these flocks due to difficulties in maintaining biosecurity during thinning (Patriarchi et al., 2011, EFSA, 2011b). Allen et al. (2008) showed that during the thinning
procedure particular *C. jejuni* strains can spread from one farm to another (nearby farm) when sharing the same bird-catching personnel and/or vehicles. However, in several other studies, no increased risk associated with this process has been shown (Russa *et al.*, 2005; Barrios *et al.*, 2006; Näther *et al.*, 2009).

1.1.4.3 *Transmission through the flock*

Once flock colonization occurs, bird-to-bird transmission within flocks is very rapid. A recent mathematical model revealed a transmission rate of 2.37 (van Gerwe *et al.*, 2009) new cases per colonized chick per day. This implies that in a flock of 20,000 broilers, the prevalence of *Campylobacter* would increase from one infected bird to 95% within the week after the first bird is infected. Indeed, in a study by Stern *et al.* (2001), the majority (95% to 100%) of birds in a flock was colonized within seven days after contact with a single (*Campylobacter*-colonized) seeder bird, regardless of the age of the animals. Drinking water and feed are believed to play an important role in this fecal-oral spread through the flock (Evans, 1992; Gregory *et al.*, 1997; Herman *et al.*, 2003; Newell & Fearnley, 2003; Sparks, 2009). Once a flock is colonized, the nipple water is often contaminated with *C. jejuni* strains that are indistinguishable from those isolated from the broilers, indicating the importance of drinking water in transmitting this zoonotic pathogen throughout the flock (Messens *et al.*, 2009).

1.1.4.4 *The effect of transportation*

Transport-induced stress has been shown to increase both the *Campylobacter* load (by 0.7 log$_{10}$ cfu/g) in broiler ceca (Stern *et al.*, 1995), as well as its excretion rates in broiler feces (by 0.8 log$_{10}$ cfu/g) after transport (Whyte *et al.*, 2001). Transport crates are often still contaminated with *Campylobacter* when reused because crate decontamination processes are mostly ineffective. Contaminated crates can lead to external contamination of birds during transport of a negative flock to the processing plant (Slader *et al.*, 2002; Herman *et al.*, 2003; Hansson *et al.*, 2005; Rasschaert *et al.*, 2007). However, there is still controversy about the possible role of transport crates in transmission of *Campylobacter*. Evidence for intestinal colonization or co-colonization due to transport in *Campylobacter*-contaminated containers was not found by Rasschaert *et al.* (2007). On the other hand, *C. jejuni* genotypes commonly associated with chickens were dominantly found on transport equipment and persisted through the decontamination process (consisting of washing with water and peracetic acid sanitization), indicating that improperly disinfected transport crates are involved in *Campylobacter* contamination of poultry flocks (Hastings *et al.*, 2010; Patriarchi *et al.*, 2011).
1.1.4.5 Combined hygienic approach to prevent flock colonization

It is clear that lots of risk factors are involved in the environmental transmission of Campylobacter to broiler flocks (table 1). Possibly these factors are intimately linked with each other. The increased temperature during summer months for instance could promote the presence of flies and rodents at the farm, while increased rain fall can create water puddle reservoirs in which C. jejuni can persist and transmit to other vectors, such as cattle and other farm animals, birds, flies and rodents (Jorgenson et al., 2011). Therefore, source attribution for Campylobacter colonization in poultry flocks is not straightforward and only a combined approach of properly implemented hygienic measures in all of these areas will be capable to significantly reduce the number of Campylobacter-colonized flocks. This combined approach must aim at minimizing the probability that Campylobacter enters the broiler room (by rodent and insect control, footbath disinfection for personnel working in the broiler room and drinking water treatment; see 1.3 for on-farm Campylobacter control measures) and preventing cross-contamination during transport (by decontamination of transport crates) and slaughter (see below).

1.1.5 Broiler carcass contamination with Campylobacter

A significant correlation exists between the Campylobacter numbers in ceca of broiler chickens during rearing and bacterial counts on their carcasses after processing (Herman et al., 2003; Rasschaert et al., 2006; Rosenquist et al., 2006; Reich et al., 2008). Worldwide, an average prevalence of Campylobacter-contaminated poultry carcasses is reported to be in the range of 60% to 80% (Suzuki & Yamamoto, 2009; EFSA, 2010c; Mullner et al., 2010). Similarly as in live animals of a poultry flock, also on poultry carcasses C. jejuni is the predominating species (Rasschaert et al., 2006; Kuana et al., 2008; Suzuki & Yamamoto, 2009; EFSA, 2010b; EFSA, 2010c, EFSA, 2012). Carcass contamination occurs during defeathering and evisceration, by contaminated feces leaking from the cloaca and visceral rupture of ceca carrying a high Campylobacter load (Berrang et al., 2001; Allen et al., 2008; Boysen & Rosenquist, 2009).

The source of the majority of Campylobacter genotypes contaminating carcasses during the slaughter process is probably the live flock (Herman et al., 2003; Rosenquist et al., 2003; Normand et al., 2008; Colles et al., 2010; EFSA, 2010d), but carcasses can also become contaminated by cross-contamination between birds within a flock and between flocks slaughtered successively (Herman et al., 2003; Rosenquist et al., 2003; Rasschaert et al.,...
2006) and even between carcasses of different flocks slaughtered in the same area (Normand et al., 2008), increasing the diversity of Campylobacter genotypes isolated from a flock after slaughter (Colles et al., 2010). Campylobacter-colonized flocks contaminate the abattoir environment upon entering the room and as a consequence Campylobacter can be isolated at all stages of the processing line (Ellerbroek et al., 2010). C. jejuni is able to survive overnight on these processing equipment surfaces, even after cleaning (using high-pressure water and alkaline-chlorinated molecules or a neutral detergent) and disinfection (using quaternary ammonium compounds combined with gluteraldehyde or with poly (hexamethylene biguanide) chlorohydrate) (Peyrat et al., 2008). Therefore, surviving strains may be a source of poultry carcass contamination of subsequent (Campylobacter-free) flocks (Newell et al., 2001; Miwa et al., 2003). Due to their stringent growth requirements, campylobacters are unable to grow in/on these carcasses and meat preparations.

1.1.6 Transmission of Campylobacter from broilers to humans

Transmission to humans most commonly occurs through consumption and handling of all kinds of foods of animal origin of which the carcasses are contaminated with Campylobacter (Berrang et al., 2001; EFSA, 2010c). In industrialized countries handling, preparation and consumption of contaminated chicken meat is considered to be the main source of infection in humans (Berndtson et al., 1992; Friedman et al., 2004). By using genetic typing methods (see 1.1.7) it was evidenced that chicken meat Campylobacter isolates can frequently be linked to human cases of campylobacteriosis. However, the overall genotypic diversity between isolates indicates that there are other sources contributing to disease in humans as well (de Haan et al., 2010; Thakur et al., 2010). Indeed, besides poultry, also non-poultry farm animals can contribute to campylobacteriosis in humans. Campylobacter colonization of such animals not only poses a risk of contamination of their carcasses at slaughter, but it can also lead to the contamination of milk and surface water at the farm, as well as colonization of broiler flocks present at these farms (Doyle & Roman, 1982; Stanley & Jones, 2003; Garrett et al., 2006; Hannon et al., 2009), which are all risk factors for transmitting C. jejuni to humans. Also direct contact with cattle, but also pets, in particular puppies with diarrhea, is a possible route of contamination (Tenkate & Stafford, 2001; de Haan et al., 2010). Cattle and their direct environment are thus important reservoirs for disease in humans (Kwan et al., 2008). Finally, also drinking water as well as raw vegetables, which can become contaminated by cross-contamination by other contaminated food products during preparation but also directly at the
farm, have been implicated as possible sources for human illness (Evans et al., 2003; Gardner et al., 2011; Verhoeff-Bakkenes et al., 2011).

1.1.7 Contribution of the chicken reservoir to campylobacteriosis in humans

Campylobacters generally do not cause large outbreaks of gastro-intestinal illness since over 90% of campylobacteriosis cases in humans are sporadic (Snelling et al., 2005). Due to the wide-spread occurrence of *Campylobacter* spp., its environmental cycle is not very well understood. Moreover, due to the possibility of cross-contamination, tracing the genuine source of *Campylobacter* infections is not straightforward. By using a genetic approach, however, Wilson et al. (2008) estimated that 97% of the number of sporadic human campylobacteriosis cases in England is attributable to animals farmed for meat, with chicken and cattle as main sources for *C. jejuni*, indicating that contaminated food products are the principle source for disease in humans. Stern & Kazmi (1989) stated already over two decades ago that a large number of *C. jejuni* serotypes from poultry can frequently be linked to human cases of campylobacteriosis. This was confirmed by later reports using also genetic typing techniques (Nielsen et al., 1997; Nadeau et al., 2002; Zorman et al., 2006; Colles et al., 2008; Gonzalez et al., 2009; Lindmark et al., 2009; Wassenaar et al., 2009; Mullner et al., 2010), together indicating that certain *C. jejuni* strains circulate between poultry and humans. The observation that during the Belgian dioxin crisis in 1999, a withdrawal of chicken meat from the market in June coincided with a 40% decrease in *Campylobacter* infections during that month (Vellinga & Van Loock, 2002), gave further strength to the hypothesis that poultry is a very important source for transmitting this pathogen to humans. Very recently, evidence of transmission by direct contact with poultry carcasses was given by Friis et al. (2010) who isolated identical strains from a poultry abattoir and a person that had developed campylobacteriosis upon entering that abattoir. Thus, there is increasing evidence that the broiler chick is a major reservoir for *C. jejuni* pathogenic to human and that broiler chicken meat contaminated with this zoonotic pathogen is the most important source of disease in humans. For the European situation it was estimated that *Campylobacter*-contaminated chicken meat would be responsible for up to 40% of human campylobacteriosis cases. The chicken reservoir as a whole might even be responsible for up to 80% of the cases, because strains from the chicken reservoir may reach humans by pathways other than food (EFSA, 2010c). As a consequence, eradicating *Campylobacter* from poultry lines could tremendously reduce the number of *Campylobacter*-mediated enteritis cases in humans. Unfortunately, no
effective, reliable intervention measure is available to date to reduce *Campylobacter* colonization in poultry (Hermans et al., 2011). In the EU, neither the overall prevalence of this pathogen in chicken retail products, nor the number of reported poultry meat consumption-related human campylobacteriosis cases have been reduced in recent years (EFSA, 2010c; 2012).
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1.2 **CAMPYLOBACTER COMMENSALISM IN CHICKENS: COLONIZATION MECHANISM AND IMMUNE RESPONSE**

Broiler chickens colonized with *C. jejuni* carry a large number of bacteria especially in their ceca (generally around $10^6$ to $10^8$ cfu/g), the predominant site of colonization (Beery *et al*., 1988; Meade *et al*., 2009). Ingestion of *C. jejuni* numbers as few as 35 cfu can be sufficient for successful colonization of chicks (Stern *et al*., 1988). Upon ingestion, the bacterium reaches the cecum and multiplies, resulting in a stable colonizing *Campylobacter* population within 24 hours after entrance (Coward *et al*., 2008; Smith *et al*., 2008). Despite carrying such high *C. jejuni* numbers in their ceca, colonization of chickens with *C. jejuni* does generally neither cause clinical illness nor changes in cecal mucosa morphology (Van Deun *et al*., 2008a; Meade *et al*., 2009b). Despite some reports of *Campylobacter*-induced diarrhea, systemic invasion, growth retardation, jejunal villus atrophy and development of a GBS-like paralytic neuropathy after feeding chicks with *C. jejuni* isolated from a GBS patient (Ruiz-Palacios *et al*., 1982; Sanyal *et al*., 1984; Sang *et al*., 1989; Lam *et al*., 1992; Li *et al*., 1996; Lamb-Rosteski *et al*., 2008), it is generally accepted that *C. jejuni* colonizes the avian gut as a commensal.

### 1.2.1 Colonization potential of *C. jejuni* isolates in poultry

#### 1.2.1.1 Colonization phenotypes

*C. jejuni* isolates can have different colonization potential (Stern *et al*., 1988; Ringoir & Korolik, 2003; Hanel *et al*., 2009). Isolates from humans have been reported to be less successful in colonizing chickens compared to poultry isolates (Korolik *et al*., 1998; Ringoir & Korolik, 2003). *C. jejuni* isolates from poultry have been divided into three colonization phenotypes. Strains of the first phenotype fail to colonize 14-day-old chickens. In the second phenotype, strains can colonize but are readily eliminated and are classified as transient. The third phenotype contains strains that show efficient and sustained colonization (Korolik *et al*., 1998; Hanel *et al*., 2009). These three colonization phenotypes were found to be stable and independent of *in vivo* passages and the amount of viable bacteria in the inoculum. Although *C. jejuni* strains did show enhanced colonization capacity/potential (i.e. the minimal infective dose required for colonization decreased) after passage through the avian gastro-intestinal (GI) tract, their colonization phenotype did not change (Ringoir & Korolik, 2003). Enhanced colonization capacity and increased virulence after *in vivo* passage through chicks has been
shown in several other studies as well (Sang et al., 1989; Cawthraw et al., 1996). This variability in colonization capacity, but the fixedness of the colonization phenotype of a given strain indicates that _C. jejuni_ genes involved in initial and sustained colonization are not identical. However, in contrast to this stable colonization phenotype, it has been previously reported that after several _in vivo_ passages a poorly colonizing isolate was able to consistently colonize chicks (Stern et al., 1988).

1.2.1.2 Genetic variation

Chicken intestinal colonization may favour genomic changes in _C. jejuni_ resulting in different _flaA_ types, ribopatterns and pulsotypes (Hanninen et al., 1999; Van Deun et al., 2007; Hanel et al., 2009). Interstrain genetic exchange and intragenomic alterations were shown to occur _in vivo_, even in the absence of selective pressure (de Boer et al., 2002). It has been demonstrated that bacteriophage genes are known to be present in the genome of _C. jejuni_ and that phages can alter pulsed field gel electrophoresis (PFGE) patterns of this bacterium (Barton et al., 2007; Clark & Ng, 2008). Both phage-dependent and -independent rearrangements of the genome result in an enormous antigenic variation among _C. jejuni_ isolates (Scott et al., 2007a, 2007b). Besides protection against phage predation, this generation of antigenic diversity may play an important role in chicken gut colonization. However, _C. jejuni_ strains that underwent rearrangements leading to resistance against virulent bacteriophages were demonstrated to be inefficient colonizers of the chick intestine (Scott et al., 2007b). There is still some controversy regarding the genomic instability of _C. jejuni_ since Nielsen et al. (2001) concluded that many strains were genetically stable as tested by riboprinting, PFGE, random amplification of polymorphic DNA (RAPD) and Penner heat-stable serotyping after _in vitro_ and _in vivo_ (through mice) passage. Moreover, Manning et al. (2001) concluded that this stability could be maintained despite exposure to various environmental conditions over long time periods and covering large distances. Also, it has been suggested that subtype pattern variations in _C. jejuni_ leading to phenotypic changes, occur only occasionally during _in vivo_ passage (Konkel et al., 2007). On the other hand, Ridley et al. (2008) observed that, although stable during single cecal colonization of one individual strain, the _C. jejuni_ genome can undergo changes upon competitive stress in the avian gut, i.e. when colonized with more than one sero- or genotype at the same time, which is referred to as co-colonization. This leads to pulsotype variants with different colonization capacities. Genetic and phenotypic diversity might play a role in the improved fitness of certain _C. jejuni_ strains to survive and colonize another host.
1.2.1.3 Co-colonization

Co-colonization is frequently detected in Campylobacter-positive flocks, which may be explained by recurring environmental exposure to the bacterium but also by genetic changes within the C. jejuni population (van de Giessen et al., 1992; Jacobs-Reitsma et al., 1995). The dominating strains are replaced throughout the colonization period, probably due to strain-specific immune responses, and it seems that this colonization pattern is mainly determined by the chicken host and not by the host microbiota (Skanseng et al., 2007; Ridley et al., 2008b). Indeed, different breeds of chicken may differ in their susceptibility to colonization with C. jejuni (Stern et al., 1990a; Boyd et al., 2005). It has been suggested that a paternal effect might be an important genetic factor influencing resistance to C. jejuni colonization in broilers (Li et al., 2008a). However, there are also other lines of evidence suggesting that external factors are responsible for the Campylobacter colonization pattern in broilers. It has been found in artificially inoculated birds that different C. jejuni genotypes may compete for colonization leading to a C. jejuni succession in broilers (Konkel et al., 2007).

1.2.2 Colonization mechanisms of C. jejuni in the chicken gut

As in the environment, also in the chicken intestine C. jejuni is likely to encounter environmental stressors compromising optimal growth (Murphy et al., 2006). The persistent colonization of the chicken GI tract by C. jejuni indicates that the bacterium harbours regulatory systems that confer protection toward a hostile environment inside the host. The mechanism by which the bacterium adapts to this “hostile” environment, resulting in successful and persistent colonization, is poorly understood. It is clear, however, that successful colonization of the chicken GI tract is a multifactorial process (Newell, 2002), in which genes involved in all areas of the colonization process of C. jejuni play a role. In the next sections, a thorough, up-to-date overview is given on identified colonization factors of C. jejuni in the chicken gut, summarized in table 2.

1.2.2.1 Multidrug and bile resistance

The Campylobacter multidrug efflux pump (CME) plays an important role in multidrug resistance in C. jejuni, mediating resistance to heavy metals and a broad range of antibiotics and other antimicrobial agents (Lin et al., 2002). It is also responsible for resistance to bile salts in the chicken intestinal tract and is therefore essential for successful intestinal colonization in chickens (Lin et al., 2003). CME is encoded by the operon cmeABC and
Literature review

consists of a periplasmic protein (CmeA), an inner-membrane efflux transporter (CmeB) and an outer-membrane protein (CmeC). Expression of cmeABC in C. jejuni is modulated by CmeR, functioning as a transcriptional repressor (Lin et al., 2002). In a cmeR mutant, one gene in particular was upregulated most compared to the wild type strain: cj0561c, encoding a putative periplasmic protein (Guo et al., 2008). It is suggested that CmeR directly inhibits the transcription of this gene. The expression of both cmeABC and cj0561c is strongly induced by bile compounds present in the chicken intestinal tract and expression of Cj0561c is increased over four-fold during chicken colonization (Woodall et al., 2005; Guo et al., 2008). Inactivation of cj0561c and loss-of-function mutation of CmeR resulted in reduced fitness of C. jejuni in chickens and impaired ability to colonize chicks, respectively (Guo et al., 2008).

Finally, a mutant in the Campylobacter bile resistance regulator (cbrR) gene, coding for the response regulator CbrR, was shown to be sensitive to bile components in vitro (Raphael et al., 2005). In addition, this mutant had reduced colonization ability in chicks indicating that also in vivo CbrR modulates resistance to bile salts in C. jejuni. Together, these observations indicate that bile salts and multidrug resistance is crucial for C. jejuni to survive in the chicken gut.

1.2.2.2 Chemotaxis

Since C. jejuni is a highly motile bacterium, chemotaxis might be an important factor promoting its migration toward favourable conditions, and thus its survival in and colonization of the intestinal mucosa. For successful chemotaxis, an intact gradient-sensing mechanism, in which adaptation has a crucial role, is indispensable. The C. jejuni genome contains genes encoding putative adaptation proteins: a methylesterase CheB and a methyltransferase CheR, which are both involved in a methylation-dependent chemotaxis pathway (Stephens et al., 2006) and together form the putative adaptation system CheBR, which is believed to be involved in the response of C. jejuni to environmental signals by modifying its chemoreceptors (Kanungpean et al., 2011). A ΔcheBR mutant was shown to have a reduced ability to colonize the chick cecum (Kanungpean et al., 2011). C. jejuni is attracted by the glycoprotein mucin, the principal constituent of mucus, and also by the bile and mucin constituent L-fucose. The amino acids aspartate, cysteine, serine and glutamate, and the salts of the organic acids citrate, fumarate, α-ketoglutarate, malate, pyruvate and succinate also act as chemoattractants (Hugdahl et al., 1988). Additionally, L-asparagine, formate and D-lactate were recently identified as attractants of C. jejuni (Vegge et al., 2009). Surprisingly, in this study, C. jejuni was not attracted to citrate and L-fucose. All these
chemicals are sensed by the transmembrane methyl-accepting chemotaxis proteins (MCP) of *C. jejuni* (Vegge et al., 2009). Hendrixson & DiRita (2004) identified 22 *C. jejuni* genes involved in colonization of the chicken GI tract. Severely affected colonization capacity particularly resulted from mutation in the determinant of chick colonization gene B (*docB*), encoding a putative MCP and alternatively called chemoreceptor transducer-like protein10 (Tlp10). DocC (Tlp4), another MCP, was important for obtaining wild type colonization levels. Finally, also Tlp1 is important for chick colonization since a *tlp1*-isogenic mutant showed reduced colonization ability (Hendrixson & DiRita, 2004; Hartley-Tassell et al., 2010). Surprisingly, all three chemoreceptors (*tlp1*, *tlp4* and *tlp10*) have been identified as being important for invasion (see further) of *C. jejuni* in chicken embryo intestinal cells (used as a model for *in vivo* invasion in chicken gut epithelial cells), but not for chemotaxis (Vegge et al., 2009). While it is clear that these factors contribute to *in vivo* colonization, their precise role in colonization requires further study. The putative accessory colonization factor (*acfB*), encoding a probable MCP protein, is highly upregulated in the chick cecum and although not important in the early stages of colonization, it cannot be ruled out that it might be involved in the persistence of *C. jejuni* in the chick cecum in the presence of a developed gut flora (Woodall et al., 2005). A number of other genes as well have been associated with *C. jejuni* chemotaxis, including the *Campylobacter* energy taxis response genes *cetA* and *cetB* (Golden & Acheson, 2002) and the chemotaxis regulatory gene *cheY*, which codes for a response regulator controlling flagellar rotation and is involved in the same signal transduction pathway as CheBR (Stephens et al., 2006). CheY shuttles between the MCP DocB and Tlp1 and the flagellar motor (Hendrixson & DiRita, 2004; Hartley-Tassell et al., 2010). A *cheY* mutant was affected in its colonization potential of the chick cecum (Hendrixson & DiRita, 2004). Also the production of the signal autoinducer AI-2 has been shown to be important for colonization (Quinones et al., 2009). Inactivation of *luxS*, the gene encoding the AI-2 biosynthesis enzyme, led to a decrease in chemotaxis toward organic acids, *in vitro* adherence to chicken hepatoma (LMH) cells and chick colonization. These observations indicate that energy taxis may be an important force in environmental navigation by *C. jejuni*, driving the organism toward optimal chemical conditions for colonization.

1.2.2.3 Flagella and motility

Intact and motile flagella are important colonization factors for *C. jejuni* in chickens (Nachamkin et al., 1993). *C. jejuni* contains one or two polar flagella. The flagellar filament consists of multimers of the protein flagellin and is attached by the hook protein FlgE to a
basal structure, embedded in the cell membrane and serving as a motor for rotation. The flagellin locus contains two adjacent genes, \textit{flaA} (encoding the major flagellin) and \textit{flaB} (encoding a minor flagellin). Both genes are independently transcribed, with the \textit{flaA} gene regulated by a $\sigma^{28}$ promoter and the \textit{flaB} gene by a $\sigma^{54}$ promoter (Nuijten et al., 1990; Hendrixson et al., 2001; Hendrixson & DiRita, 2004). Environmental and chemotactic stimuli modulate \textit{flaA} and \textit{flaB} promoter activity. Medium pH, growth temperature and the concentration of certain inorganic nutrients affect \textit{flaB} promoter activity (Alm et al., 1993). Lower pH, bovine bile, deoxycholate, L-fucose, high osmolarity and chemotactic effectors such as aspartate, glutamate, citrate, fumarate, $\alpha$-ketoglutarate and succinate all upregulate the \textit{flaA} promoter. Proline, high viscosity and milk fermented by \textit{Bifidobacterium} or \textit{Lactobacillus} strains downregulate the \textit{flaA} promoter (Allen & Griffiths, 2001; Ding et al., 2005). The \textit{flaA} gene seems to be highly conserved among \textit{Campylobacter} isolates and transcription is usually higher than that of \textit{flaB} (Guerry et al., 1990). Transcription of $\sigma^{54}$-dependent genes, necessary for assembly of the hook-basal body filament structure, is regulated by a two-component system composed of the sensor kinase FlgS and the response regulator FlgR (Wosten et al., 2004). Experiments with mutants have shown that \textit{flaA} but not \textit{flaB} is essential for colonization of chickens (Wassenaar et al., 1993; Jones et al., 2004) although probably both are needed for full motility (Neal-McKinney et al., 2010).

Colonization is also impaired with the mutant for the motility accessory factor 5 (maf5) gene, important for the formation of flagella (Karlyshev et al., 2002; Jones et al., 2004). Once \textit{C. jejuni} reaches the cecum, it seems that mutants in the flagellar biosynthesis genes \textit{rpoN} (encoding $\sigma^{54}$) and \textit{fliA} (encoding $\sigma^{28}$) and the response regulator gene \textit{flgR} could establish colonization at a high inoculation dose, albeit bacterial numbers were much lower compared to the controls and the number of chicks colonized with these mutants was extremely low (Hendrixson & DiRita, 2004; Wosten et al., 2004; Fernando et al., 2007). Chickens exposed to the \textit{flgR} mutants showed a delayed colonization and transmission to \textit{Campylobacter}-free chickens was not observed. However, comparable bacterial counts were detected in the ceca of birds two weeks after inoculation with either the \textit{flgR} mutant or the wild type strain. Since bird-to-bird transmission in flocks is generally considered to be very rapid, this indicates that the FlgS/FlgR system is mainly required for initial colonization and less for survival and persistence in the cecum of chicks (Wosten et al., 2004). Also the \textit{flgK} mutant, expressing only the hook, showed diminished motility and was completely attenuated for colonizing the chick cecum (Fernando et al., 2007). Further supporting indications that flagella are important colonization factors for \textit{C. jejuni} in chickens was given by Hiett et al. (2008). These authors
demonstrated differential expression patterns of flagella proteins between a poor and a robust colonizer strain in poultry. These differentially expressed genes, coding for proteins involved in the modification of the flagellum, are located in hypervariable regions of the \textit{C. jejuni} genome. This variability was shown to be extendable to the protein level, and thus may contribute to the survival of \textit{C. jejuni} in its different environments and hosts.

In \textit{C. jejuni} chicken isolates, the flagellin \textit{O}-linked glycosylation island, responsible for successful flagellin assembly and motility, is very diverse (Karlyshev \textit{et al.}, 2005). Five genes (\textit{cj1321} - \textit{cj1325/6}) lying in this variable region are, however, significantly prevalent among \textit{C. jejuni} strains associated with poultry (Champion \textit{et al.}, 2005) and might therefore be important for the ability of certain \textit{C. jejuni} strains to colonize this host. Mutagenesis and functional and structural data supported this hypothesis, with particularly \textit{cj1324} being important for chick colonization (Howard \textit{et al.}, 2009).

The flagellar apparatus functions as a type III secretion apparatus for the \textit{Campylobacter} invasion antigens (Cia proteins) (Konkel \textit{et al.}, 2004), important for \textit{in vitro} cell invasion (Konkel \textit{et al.}, 1999) and chick colonization (Ziprin \textit{et al.}, 2001), and secretion is enhanced upon exposure to chicken mucus (Biswas \textit{et al.}, 2007). A correlation has been demonstrated between chicken colonization potential and \textit{in vitro} secretion of Cia proteins (Biswas \textit{et al.}, 2007). \textit{RpoN} mutants are completely aflagellated and as such do not secrete Cia proteins, nor do \textit{flgK} mutants (Fernando \textit{et al.}, 2007), making it clear that the molecular basis behind the colonization mechanism in chickens is complex.

The role of motility of \textit{C. jejuni} colonization in the chicken GI tract is not fully understood. Non-motile \textit{C. jejuni} mutants can colonize chickens, be it at substantially reduced levels and only when chickens are inoculated with high amounts of viable cells (Wosten \textit{et al.}, 2004). Probably, motility is needed for \textit{C. jejuni} to pass the GI tract so it can reach its protective niche, the mucus layer of the cecal crypts (Beery \textit{et al.}, 1988), and to resist gut peristalsis (Hendrixson & DiRita, 2004), hence it is important for initial colonization. It is, however, not known if motility is important in the persistence of \textit{C. jejuni} in the intestinal tract, leading to long-term colonization. In any case, it is clear that the specialized flagellum of \textit{C. jejuni} serves multiple functions in the adaptation of \textit{C. jejuni} to the chicken GI tract.

1.2.2.4 \textit{Surface-accessible carbohydrate structures and immune evasion}

Several surface-accessible carbohydrate structures (SACS) such as flagella, lipooligosaccharides (LOS), a capsule and \textit{O}- and \textit{N}-linked glycans contribute to \textit{C. jejuni} colonization in chicks.
In *C. jejuni*, the lipopolysaccharide molecule only consists of lipid A and the (inner and outer) core oligosaccharide and is therefore referred to as LOS, as the high-molecular-weight *O*-polysaccharide is a capsular polysaccharide not linked to the lipopolysaccharide molecule (Karlyshev et al., 2000). *C. jejuni* LOS is important for host cell adhesion and invasion and chick colonization, and sialylation of the LOS outer core further enhances epithelial cell invasion (Louwen et al., 2008; Javed et al., 2012). Moreover, sialylated LOS results in reduced immunogenicity (Guerry et al., 2000) and increased invasion potential in Caco-2 cells (Habib et al., 2009). The majority of strains from human and chicken origin belonging to the clonal complex CC-21, an ecologically diverse and the largest complex in the general population structure of *C. jejuni*, were found to belong to one sialylated LOS class in particular, LOS class C, correlating with a high invasive potential (Habib et al., 2009). Thus, sialylation of the LOS outer core is likely to contribute to successful colonization of *C. jejuni* in a suitable host. Genes responsible for the formation of the polysaccharide capsule, surrounding the surface of *C. jejuni* cells and possibly involved in survival, adherence and evasion of the host’s immune system (Roberts, 1996; Karlyshev et al., 2000), also play a role in colonization of the chicken intestine by *C. jejuni*. Mutation in the capsular polysaccharide transporter gene M (*kpsM*), which results in the loss of a high molecular weight glycan, and thus absence of a capsule, abolished colonization of chickens (Jones et al., 2004; Bacon et al., 2001). A *C. jejuni* mutant for the *kpsE* gene, which is unable to express any capsular polysaccharide, was not hampered in its ability to colonize the chicken intestinal tract but the number of bacteria recovered from cecum and colon were lower compared to the control (Bachtiar et al., 2007). Interpretation of these results is hampered by the use of different chicken *in vivo* models and bacterial strains. Capsule formation and LOS biosynthesis genes are located in hypervariable regions in the *C. jejuni* genome (Parkhill et al., 2000), resulting in an enormous antigenic diversity among isolates.

*C. jejuni* is unique in being the only known prokaryote having an *N*-linked protein modification system, which is encoded by the *pgl* multigene locus (Kakuda et al., 2006; Young et al., 2007). The *N*-linked glycosylation pathway is responsible for post-translational modification of multiple proteins, including flagellin, and is conserved among *C. jejuni* isolates (Szymanski et al., 1999; Karlyshev et al., 2005). In contrast, the only known proteins to be modified by *O*-linked glycosylation in *C. jejuni* (see above) are flagellar subunits (Karlyshev et al., 2005). In humans, most of the *N*-linked glycosylated proteins are highly immunogenic with their glycosyl moieties being immunodominant while only limited antibody is generated against the protein fraction (Szymanski et al., 1999). This indicates that
glycosylation might offer *C. jejuni* a unique system of immune evasion by masking primary amino acid sequences. A mutant in the *N*-linked general protein glycosylation pathway gene H (*pglH*) possessed an intact capsule, but was unable to glycosylate proteins and was severely reduced in its ability to colonize the chicken intestinal tract (Jones *et al*., 2004; Karlyshev *et al*., 2004). Also strains with other mutations in the *pgl* locus were affected in their ability to colonize chicks (Hendrixson & DiRita, 2004), indicating that *N*-linked glycosylation in *C. jejuni* is an important colonization determinant. However, glycan modification of Cj1496c, a glycoprotein important for *in vitro* cell invasion in human epithelial cells and initial chick colonization does not seem to influence its function (Kakuda *et al*., 2006). Moreover, most *N*-glycosylated proteins, including Cj1496c, are annotated to be periplasmatic and do not come in direct contact with host factors and the exact mechanism by which this glycosylation system contributes to colonization remains to be elucidated (Young *et al*., 2002; Kakuda *et al*., 2006).

To conclude, several SACS of *C. jejuni*, including the unique *N*-linked glycans, contribute to successful colonization in chicks. Not only by mediating adhesion (see further), but also by creating an enormous antigenic diversity in *C. jejuni* isolates resulting in persistent high-level gut colonization of certain strains.

1.2.2.5 Two-component regulatory systems

*C. jejuni*, like all prokaryotes, responds to environmental changes by using two-component regulatory systems (TCRSs) consisting of sensor (S) kinases and response (R) regulators, regulating *C. jejuni* gene expression (Woodall *et al*., 2005; Mikkelsen *et al*., 2011). A histidine kinase senses specific environmental triggers through autophosphorylation of the histidine residue. Subsequent transfer of the phosphate group to the corresponding response regulator turns it into an active transcription factor that can stimulate the differential expression of target genes, allowing *C. jejuni* to immediately respond to changing environmental conditions (such as several stressors, nutrients and temperature) within the chicken gut (Mikkelsen *et al*., 2011).

To date, five TCRSs have been identified in *C. jejuni* to be important for optimal chick colonization: FlgRS (Wosten *et al*., 2004) and the orphan response regulator CbrR (Stephens *et al*., 2006) (see above), the reduced ability to colonize (RacRS) system (Bras *et al*., 1999), diminished capacity to colonize (DccRS) (MacKichan *et al*., 2004) and *Campylobacter* planktonic growth regulation (CprRS) (Svensson *et al*., 2009). RacRS is responsive to temperature, and both mutation of racR (Bras *et al*., 1999; Woodall *et al*., 2005) or RacS
(Apel et al., 2012) reduce the colonization potential of C. jejuni (see below). DccRS controls the expression of several genes encoding probable membrane-associated proteins (Woodall et al., 2005). Finally, CprRS is thought to control essential biological processes, stress tolerance and biofilm formation, making it possible for C. jejuni to adapt to different environments (Svensson et al., 2009). A ΔcprS mutant was reported to display a dramatic dose-dependent defect for chick colonization. Thus, it is clear that the genome of C. jejuni harbours multiple TCRS genes, involved in all aspects of C. jejuni biology, which are vital for its efficient adaptation to the chicken host.

1.2.2.6 Temperature regulation and heat shock response

The elevated body temperature of the chicken (42°C) as compared to humans implies the transcription of many different proteins uniquely transcribed in response to the chicken GI tract environment. The RacR/RacS signal transduction system responds to temperature changes and might play an important role in chicken colonization with C. jejuni (Bras et al., 1999). Comparative analysis of the protein profile of wild type C. jejuni and racR mutants, revealed 11 proteins to belong to the RacR regulon. Three proteins were sequenced and were identified as RacR and two isoforms of a cytochrome c peroxidase homologue. A comparative study by Zhang et al. (2009) revealed 15 to 20 proteins differentially expressed by at least two-fold when C. jejuni was grown at 37°C or at 42°C. All identified, differentially expressed proteins are periplasmic proteins or major antigens of C. jejuni, or are involved in the metabolism or regulatory system. These proteins might play a role in adaptation to and pathogenicity in the different hosts of C. jejuni. It was demonstrated that RacR is important for growth at 42°C (Bras et al., 1999). DnaJ belongs to a family of heat shock proteins and plays a role in C. jejuni thermostolerance (Konkel et al., 1998). Mutation of dnaJ severely reduced colonization in chicks (Konkel et al., 1998; Ziprin et al., 2001). The dnaJ gene is located adjacent to racR and under the transcriptional control of RacR (Bras et al., 1999; Apel et al., 2012). While racRS is activated, dnaJ is repressed by RacR.

1.2.2.7 Adhesion

Campylobacter adhesion to epithelial cells of the chicken GI tract is believed to be an important step in successful colonization. Several studies contributed to the importance of intact flagella and adhesins, surface-exposed proteins, in chicken colonization. Mutation of the Campylobacter adhesion protein A (capA) gene, encoding an autotransporter lipoprotein, resulted in reduced capacity to adhere to human and chicken intestinal epithelial cells and
reduced invasion capacity in human epithelial cells (Ashgar et al., 2007; Flanagan et al., 2009). While capA mutation abolished colonization in a chick model in the study by Ashgar et al. (2007), Flanagan et al. (2009) did not observe reduced colonization capacity for the capA mutant. Moreover, since this gene is absent in many C. jejuni poultry isolates, the genuine contribution of capA to successful chick colonization is unclear (Flanagan et al., 2009; Friis et al., 2010). The Campylobacter adhesion to fibronectin (CadF) outer-membrane protein (OMP) was shown to bind to fibronectin, a glycoprotein of the extracellular matrix of the GI tract (Konkel et al., 1997), and to be important for full binding capacity of C. jejuni to chicken epithelial cells (Flanagan et al., 2009). Ziprin et al. (1999, 2001) demonstrated that mutants in the genes cadF and pldA, the structural gene for phospholipase A, are impaired in their ability to colonize the cecum, indicating that these genes may play a prominent role in successful colonization. However, in contrast to the highly prevalent cadF gene, many C. jejuni isolates lack the pldA gene (Rizal et al., 2010). Moreover, the biological function of pldA is not known, but due to its outer-membrane localization it might be involved in maintaining the functional integrity of surface exposed adhesins in some strains (Dekker, 2000). Hiett et al. (2008) demonstrated differential expression patterns for major OMPs in poultry between a poor and a robust colonizer strain. These differentially expressed genes are located in hypervariable regions of the C. jejuni genome and may contribute to the survival of C. jejuni in its different environments and hosts. Recently, a new adhesin, fibronectin-like protein A (FlpA), has been identified to be important for full binding capacity to chicken epithelial cells and successful colonization (Flanagan et al., 2009). Konkel et al. (2007) found that different C. jejuni strains compete for colonization in broilers and hypothesized that this is due to the sharing of common adhesins among these isolates and limited host epithelial cell binding places. This finding supports the hypothesis that adhesion is a key step in the colonization process of C. jejuni in chicks.

1.2.2.8 Invasion

Invasion might be an important colonization determinant of C. jejuni in chicks because mutations in ciaB as well as in the MCP genes tlp1, tlp4 and tlp10, important for in vitro invasion in mammalian and chicken cells respectively (see above), severely impair cecal colonization (Ziprin et al., 2001; Vegge et al., 2009). Studies with isolated primary intestinal cells from chickens indeed showed that C. jejuni was able to invade chicken cells (Byrne et al., 2007; Li et al., 2008; Van Deun et al., 2008b), an unexpected feature since C. jejuni does not associate with chicken crypt epithelium in vivo (Byrne et al., 2007). Invasion capacity was
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largely strain-dependent, but overall no difference was observed between isolates from poultry or human origin. Microtubule- as well as microfilament-dependent invasion was reported, which is in accordance with results obtained from invasion experiments in human epithelial cell lines (Hu & Kopecko, 1999). Many studies on the genes which are thought to play a role during invasion have been conducted on human epithelial cell lines, but thus far experiments on chicken primary epithelial cecal cells are lacking. While it is tempting to assume that invasion mechanisms in these cells are analogous to those in human cell lines, some differences do exist: C. jejuni can survive in vitro in human T84 epithelial cells by avoiding fusion with lysosomes (Watson et al., 2008), but intracellular survival seems not to be the case in the primary chicken enterocytes (Van Deun et al., 2008b). The lack of an immortalized chicken intestinal cell line and the complicated handling of primary chicken cecal cells clearly hamper investigation toward invasion (and other) mechanisms in chicken cecal cells. Nevertheless, the recent obtained in vitro and in vivo results described under this section suggest that invasion of C. jejuni in gut epithelial cells might be an important colonization determinant in vivo.

1.2.2.9 Iron transport and regulation

Regulation of the intracellular iron concentration is an important factor to secure colonization. Iron is essential for electron transfer processes and functions as a cofactor for several enzymes. It is also responsible for the generation of hydroxyl radicals. Moreover, iron availability modulates the transcription of genes belonging to several functional groups, thereby affecting the ability of C. jejuni to colonize the GI tract (Palyada et al., 2004). The soluble ferrous iron (Fe^{2+}) is readily transported across the outer-membrane via porins and is subsequently transported across the cytoplasmic membrane by a specific transporter protein, FeoB. This transporter is important for iron acquisition and intracellular survival of C. jejuni, as well as for successful gut colonization (Naikare et al., 2006). Mutants in the ferric uptake regulator (fur) gene, the cfrA gene responsible for an outer-membrane ferric enterobactin (FeEnt) receptor and the ceuE gene encoding a FeEnt periplasmic binding protein regulated by fur, are all compromised in their ability to colonize chickens, with complete absence of live bacteria for the latter two (Palyada et al., 2004), as were mutants in another recently identified and characterized outer-membrane FeEnt receptor CfrB, which is most prevalent in C. coli strains (Xu et al., 2010). Inactivation of cfrB in a cfrA-negative C. jejuni strain fully abolished its ability to utilize FeEnt as a sole iron source for growth. Moreover, the reduced colonization phenotype of the isogenic cfrB mutant of C. jejuni could not be restored by the
presence of a functional cfrA gene. In contrast, complementation of an isogenic cfrA mutant with the wild type cfrB gene in trans fully restored the ability of this _C. jejuni_ mutant to utilize FeEnt. Thus, CfrB plays an important role during colonization of _Campylobacter_ in chicks and cannot be compensated by other iron uptake mechanisms without affecting the colonization potential. Therefore, it is believed that CfrB is the relevant receptor for FeEnt utilization during colonization of chickens with _C. jejuni_ strains producing both a functional CfrA and CfrB. Transcription levels of chuA, a gene believed to code for an outer-membrane receptor for hemin and hemoglobin, are increased over 40-fold in the chicken cecum, indicating that ChuA might be required for _C. jejuni_ to colonize chicks (Woodall et al., 2005). Finally, mutation in Cj0178, a putative transferrin-bound iron utilization outer-membrane receptor, resulted in reduced colonization potential (Palyada et al., 2004). Given this information, it can be concluded that several iron-uptake systems are essential for the survival of _C. jejuni_ and for its successful colonization in the chicken host.

Besides iron, also zinc has been reported to be an important trace element necessary for _C. jejuni_ growth inside the chicken host (Davis et al., 2009). A _C. jejuni_ mutant lacking ZnuA, the periplasmic component of a putative zinc ATP-binding cassette (ABC) transport system, had a growth defect in zinc-limiting media and was severely affected in its colonization potential in chickens.

### 1.2.2.10 Oxidative and nitrosative stress defense

_C. jejuni_ is a microaerophilic microorganism and thus requires reduced oxygen levels for its growth. Nevertheless, it must resist oxidative stress it may encounter both in the environment and in its host, like the superoxide anion, hydrogen peroxide and biotoxic hydroxyl radicals. These stressors can result from incomplete reduction of oxygen by _C. jejuni_, or be induced by the chick immune system (Atack & Kelly, 2009). _C. jejuni_ contains a wide range of enzymes involved in defense against oxidative stress. Several of these regulators have already been identified. However, the mechanism of gene regulation in _C. jejuni_ is still poorly understood. Cytochrome c peroxidases (CcPs) are generally responsible for the conversion of hydrogen peroxide to water (Atack & Kelly, 2009). With a technique called subtractive hybridization, Ahmed et al. (2002) found 23 DNA sequences, including cytochrome oxidase III, to be present in a robust colonizer but absent from a poor colonizer _C. jejuni_ strain. No direct link could be found that these factors correlate with the identified genes by Hendrixson & DiRita (2004), but it can be assumed that also these strain-specific genes are factors important for efficient and sustained colonization. _C. jejuni_ has two CcP loci, which surprisingly do not
contribute to hydrogen peroxide resistance and thus do not protect against oxidative stress. Instead, it seems that, in *C. jejuni*, resistance to hydrogen peroxide is mainly mediated by the sole cytoplasmic catalase KatA, breaking it down to water and oxygen (Bingham-Ramos & Hendrixson, 2008; Atack & Kelly, 2009). Nevertheless, mutation in one of the two CcP loci, *docA*, located immediately upstream of *docB*, resulted in a substantial dose-dependant decrease in colonization potential (Hendrixson & DiRita, 2004; Bingham-Ramos & Hendrixson, 2008). Moreover, Woodall *et al.* (2005) found Cj0358, another putative CcP, to be upregulated 12-fold *in vivo* suggesting a role for this protein in hydrogen peroxide removal from the periplasm. By constructing an isogenic Δ*perR* mutant, deficient in the regulon of the peroxide-sensing regulator (PerR), and comparing its transcriptome profile with that of the wild type strain, Palyada *et al.* (2009) identified over 100 genes to be part of the PerR regulon. Mutation of *perR* significantly reduced *C. jejuni* motility and attenuated colonization in chickens. This study also revealed a functional network between the key players of the oxidative stress defense system, including mainly the antioxidant proteins encoded by the superoxide dismutase (*sodB*), defending *C. jejuni* against the superoxide anion, the alkyl-hydroperoxide reductase (*ahpC*) and *katA*, their transcriptional regulators *fur* and *perR* and the regulatory pathways that connect them. This indicates that there is a link between oxidative stress (PerR-regulated) and iron metabolism (Fur-regulated) in *C. jejuni*. Indeed, it was recently shown that transcription of *perR* is reduced in the presence of iron (Kim *et al.*, 2011). Moreover, it seems that oxidative stress defense mechanisms and their proper regulation are essential for successful and efficient colonization of the chick cecum. Indeed, the colonization potential in chicks was reduced by 50,000-fold in the *C. jejuni ΔahpC* mutant, while colonization in Δ*perRΔfur*, ΔkatA and Δ*sodB* mutants was completely abolished. This indicates that all key players of this functional network need to be intact for successful colonization of *C. jejuni* in chicks. Gareniaux *et al.* (2008) demonstrated that next to KatA, CadF and FlaA also a periplasmic protein (Cj1371) was overexpressed following exposure to paraquat, a strong oxidizing agent. In contrast, levels of SodB were unaltered, while the *Campylobacter* oxidative stress regulator CosR was downregulated by paraquat, but not by hydrogen peroxide (Hwang *et al.*, 2011). CosR negatively regulates SodB and LuxS and positively regulates AhpC (Hwang *et al.*, 2011). In addition, a *cj1371* mutant and a CosR knockdown were both found to be more sensitive to oxidative stress (Gareniaux *et al.*, 2008; Hwang *et al.*, 2011). This indicates that they play a role in *C. jejuni* oxidative stress resistance and might be important for persistent chick colonization, but this has yet to be demonstrated.
The enzyme $\gamma$-glutamyl transpeptidase (GGT) is involved in maintaining cellular glutathione levels. Glutathione is an antioxidant molecule providing vital cellular protection against reactive oxygen species, generated by aerobic respiration (Tate & Meister, 1981; O'Donovan & Fernandes, 2000). GGT was shown to be present in a robust but absent from a poor colonizer $C.\ jejuni$ strain (Ahmed et al., 2002), suggesting that GGT activity is not needed for initial colonization but indispensable for persistence of $C.\ jejuni$ in the avian gut (Barnes et al., 2007). GGT catalyzes the conversion of glutathione and glutamine to glutamate, and the ability of certain $C.\ jejuni$ strains to utilize glutamine or glutathione as a sole carbon source is absolutely dependent on the presence of GGT (Hofreuter et al., 2008). GGT is not present in all $C.\ jejuni$ strains (Barnes et al., 2007) which could explain the lower colonization capacity of strains lacking a functional GGT.

A $ppk1$ and $ppk2$ mutant, defective in respectively polyphosphate kinase 1 (PPK1) and 2 (PPK2), two key enzymes of the polyphosphate metabolism, were shown to have decreased invasion ability in human intestinal epithelial cells and a dose-dependent colonization defect in chicken ceca (Candon et al., 2007; Gangaiah et al., 2010). This indicates that the utilization and accumulation of polyphosphate helps $C.\ jejuni$ to adapt to the cecal environment of the chick.

For survival and optimal colonization in the chick, $C.\ jejuni$ must also be capable of eliciting a suitable response to cytotoxic nitric oxide (NO), a free radical produced by several cells of the host immune system that is bactericidal against $C.\ jejuni$ (Shepherd et al., 2011). $C.\ jejuni$ is protected against NO-induced nitrosative stress by NO-detoxifying mechanisms, including a nitrite reductase and its single-domain $Campylobacter$ globin (Cgb) (Pittman & Kelly, 2005; Smith et al., 2011). Expression of Cgb in response to NO is neither regulated by Fur nor PerR, but mediated by the transcription factor NssR, regulating a nitrosative stress response regulon that also includes a truncated haemoglobin (Ctb), probably involved in oxygen metabolism (Tate & Meister, 1981; Elvers et al., 2005). NO detoxification in $C.\ jejuni$ is believed to proceed via a Cgb-catalyzed dioxygenase or denitrosylase reaction, converting NO and oxygen to nitrate (Shepherd et al., 2011).

Many $C.\ jejuni$ redox proteins essential for electron transfer (see further) have N-terminal twin-arginine translocase (TAT) signal sequences ensuring proper transport across the cytoplasmic membrane (Hitchcock et al., 2010). The TAT secretion system has been shown to be important for $C.\ jejuni$ to cope with stress and for chick colonization (Rajashekara et al., 2009). A $C.\ jejuni$ tatC knockout mutant had defects in biofilm formation, motility and flagellation, and was defective in survival under osmotic shock and oxidative and nutrient
stresses, impairing the efficient transmission of \textit{C. jejuni} to a susceptible host. The \textit{ΔtatC} mutant was unable to persistently colonize chickens which is likely the result of multiple, additive effects caused by the inability of the \textit{tatC} mutant to translocate essential TAT substrates (Rajashekara \textit{et al.}, 2009). Also a \textit{cj0379c} mutant, lacking a functional TAT translocated molybdo-enzyme of unknown function, was deficient in chick colonization (Hitchcock \textit{et al.}, 2010). The nitrosative stress phenotype of this mutant suggests a role for Cj0379 in the reduction of reactive nitrogen species in the periplasm.

It is clear that within its chicken host, \textit{C. jejuni} can encounter several stressors which it must resist for successful colonization. The evidence above indicates that \textit{C. jejuni} developed some interplaying survival mechanisms that allow the organism to cope with chicken gut-induced oxidative and nitrosative stress.

1.2.2.11 \textit{Central intermediary and energy metabolism} 

In \textit{C. jejuni}, all enzymes necessary for a complete oxidative tricarboxylic acid cycle are present. A key step in this cycle is the oxidation of succinate to fumarate. Until recently, it was believed that in \textit{C. jejuni} this reaction is exerted by both a fumarate reductase (Frd) and a succinate dehydrogenase (Sdh), since both enzymes were found to contribute to the total fumarate reduction of \textit{C. jejuni} \textit{in vitro} and were significantly upregulated in the chick cecum (Woodall \textit{et al.}, 2005; Weingarten \textit{et al.}, 2009). A \textit{C. jejuni} mutant missing the intact FrdA subunit of the FrdABC enzyme was completely deficient in its succinate dehydrogenase activity \textit{in vitro} and had reduced colonization ability in chicks. In contrast, experiments with the \textit{sdhA} mutant of \textit{C. jejuni} showed that Sdh exhibits no succinate dehydrogenase activity and is not required for colonization, indicating that the \textit{sdh} operon has been misannotated. Thus, Frd is the sole succinate dehydrogenase of \textit{C. jejuni} and is therefore essential for full host colonization (Weingarten \textit{et al.}, 2009).

To meet all of its energy demands, \textit{C. jejuni} utilizes oxidative phosphorylation (Weerakoon \textit{et al.}, 2009). In the chicken cecum, however, \textit{C. jejuni} encounters an environment with reduced oxygen levels to which it must elicit a suitable response to efficiently and persistently colonize this part of the gut. Microarray analysis revealed several genes involved in this response to be upregulated when \textit{C. jejuni} enters its host compared to \textit{in vitro} culture (Woodall \textit{et al.}, 2005), with three genes in particular: the anaerobic C\textsubscript{4}-dicarboxylate transporter genes \textit{dcuA} and \textit{dcuB} as well as the aspartase gene \textit{aspA}. Probably these genes play an important role during chick colonization. A double mutant in hydrogenase (Hyd) and formate dehydrogenase (Fdh) and a mutant in 2-oxoglutarate:acceptor oxidoreductase (OoR),
had markedly reduced colonization ability in chicks, indicating the importance of these electron donor enzymes (Weerakoon et al., 2009). The same authors also identified NADH:ubiquinone oxidoreductase (complex I) to play an important role because a mutant in this gene showed impaired colonization capacity. Mutants in the respiratory enzymes nitrate reductase, nitrite reductase and cbb$_3$-type oxidase all colonize the chicken cecum to a lesser extent (Weingarten et al., 2008). Moreover, these enzymes are upregulated in the chick cecum, indicating that C. jejuni might utilize nitrite and nitrate, as well as fumarate as a terminal electron acceptor instead of oxygen (Woodall et al., 2005). Especially nitrate is considered as a potential in vivo electron acceptor (Pittman & Kelly, 2005). It is suggested that the ability of C. jejuni to use gluconate as an electron donor is important for full colonization potential in the avian host (Pajaniappan et al., 2008). A cj0415 mutant, lacking gluconate dehydrogenase (GADH) activity, was impaired in establishing colonization in chicks but not in mice, which can probably be explained by the higher expression level of cj0415 at 42°C compared to 37°C (Pajaniappan et al., 2008).

C. jejuni is an asaccharolytic bacterium and is therefore entirely dependent on a tight set of amino acids including L-aspartate, L-glutamate, L-proline and L-serine and Krebs cycle intermediates as a primary carbon and energy source (Kelly, 2008). Mutants of the L-serine dehydratase gene sdaA were defective to catabolize L-serine and their colonization potential in chicks was abolished (Velayudhan et al., 2004). Moreover, sdaA was upregulated more than two-fold in C. jejuni upon colonizing the chick cecum, indicating the importance of serine for in vivo survival (Woodall et al., 2005). Also aspA has been demonstrated to be upregulated (by 4.8-fold) in the chick cecum (Guccione et al., 2008). An aspA mutant, which was unable to use any amino acid besides L-serine, was shown to have impaired ability to persist in the intestines of outbred chickens, which can possibly be explained by the reduced growth potential of this mutant in the avian gut, because aspartate enhances oxygen-limited growth of C. jejuni in an AspA-dependent way. Also mutation in livJ, livK and cj0903c, genes involved in amino acid transportation in C. jejuni, resulted in a marked colonization defect in chicks, but their precise respective biological functions are not fully understood (Hendrixson & DiRita, 2004; Ribardo & Hendrixson, 2011).

Due to an observed reduction in adhesion to and invasion in cultured epithelial cells the PEB1a protein has been regarded as a putative adhesin (Leon-Kempis et al., 2006; Flanagan et al., 2009). A peb1A mutant was not capable of colonizing chicks but did, however, not show a reduced binding capacity to chicken LMH cells (Flanagan et al., 2009). This suggests that PEB1a serves a role other than, or next to, mediating adhesion during in vivo
colonization. Indeed, this trans-membrane protein is believed to function as an ABC transporter of aspartate and glutamate, essential for the utilization of these amino acids as a carbon source during microaerobic growth (Leon-Kempis et al., 2006; Flanagan et al., 2009; Gangaiah et al., 2010). However, the two-component signal peptide of PEB1a might be responsible for its localization both in the periplasm as on the cell surface, where it could act as an adhesin (Leon-Kempis et al., 2006). It is unclear whether PEB1a is present in the outer-membrane, but it can definitely be found in the supernatant of C. jejuni cultures, indicating that the protein can be exported across the outer-membrane. Nevertheless, no direct evidence is available that PEB1a functions as an adhesin in C. jejuni. Therefore, the inability of the peb1A mutant to colonize chicks (Flanagan et al., 2009) is probably attributable to the inability of this mutant to utilize glutathione, glutamine and the dipeptide γ-glutamylcysteine, although GGT activity is not affected. Thus, GGT allows the utilization of these nutrients by generating glutamate, which is then taken up by the PEB1a-dependent transporter and subsequently used as a carbon source (Hofreuter et al., 2008).

To conclude, due to its assaccharolytic and microaerophylic nature, C. jejuni is dependent on amino acids and electron acceptors other than oxygen as primary energy sources for optimal growth. Although the underlying mechanisms are not yet fully characterized, several of the key molecules and genes of the central intermediary and energy metabolism have been identified. It is clear that disturbance in the proper metabolism of these nutrients is accompanied by a severely hampered survival potential and colonization ability in chicks.
<table>
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<tr>
<th>Functional area</th>
<th>Gene name or locus</th>
<th>Identified/predicted protein function</th>
<th>Reference</th>
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<td>Campylobacter multidrug efflux pump</td>
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<td>transcriptional repressor of cmeABC</td>
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<td>cj0561c</td>
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<td>cbrR</td>
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<td>signal autoinducer AI-2 biosynthesis enzyme</td>
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<td>cheB, cheR</td>
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<td>rpoN</td>
<td>RNA polymerase $\sigma^{54}$ subunit</td>
<td>Hendrixson &amp; DiRita, 2004; Fernando et al., 2007</td>
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<td>fliA</td>
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<td>flgR</td>
<td>response regulator</td>
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* Underlined and bold: genes having (probable) multiple functions
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<tr>
<th>Category</th>
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<th>Function</th>
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### Literature review

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<td>ggt</td>
<td>γ-glutamyl transpeptidase</td>
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1.2.3 Chicken intestinal immune response against *C. jejuni*

Upon entering the chicken GI tract, *C. jejuni* is attracted to the mucus layer by chemoattractants sensed by MCPs. Generally, the host intestinal mucus layer that lines the epithelial cells prevents most commensal bacteria to make direct contact with the epithelial surface by constituting a viscous physical barrier and by harbouring secretory IgA and antimicrobial peptides (Ivanov & Littman, 2011). However, although increased viscosity has been associated with down-regulation of flaA promoter activity in *C. jejuni* (Allen *et al*., 2001), its modified flagellum allows the bacterium to penetrate the viscous mucus layer (Guerry, 2007) and to reach and make contact with the intestinal epithelial cells. Although *C. jejuni* is not found to be attached to chicken cecal crypt microvilli *in vivo* (Beery *et al*., 1988), the bacterium has been observed intracellular in intestinal epithelial cells of experimentally inoculated three-day-old chickens and in chicken primary cecal epithelial crypt cells *in vitro* (Van Deun *et al*., 2008b). *C. jejuni* adherence and invasion of cells of the chicken gut epithelium is initially followed by an inefficient innate immune response by the chick, eventually resulting in the production of specific antibodies (Larson *et al*., 2008; Shoaf-Sweeney *et al*., 2008). Such a response is not able to clear *C. jejuni* from the gut since most flocks that become colonized stay so until slaughter (Jacobs-Reitsma *et al*., 1995; Stern *et al*., 2001b; Stern, 2008; Van Gerwe *et al*., 2009). Although not all birds in a flock were colonized, it was demonstrated that *C. jejuni* can be isolated from laying hens until an age of 42 weeks (Lindblom *et al*., 1986) and probably longer, since experimental periods exceeding one year are not documented. This implies that *C. jejuni* can evade the chicken host immune system for an extended period of time. However, reduced bacterial counts in *Campylobacter*-colonized chicks, as well as a reduction in the number of colonized chicks has been observed (Sahin *et al*., 2003; Cawthraw & Newell, 2010), indicating some mucosal clearance. In addition, with older birds it cannot be ruled out that replacement of one *C. jejuni* strain by an immunologically distinct strain (strain succession) occurred, disguising mucosal clearance of the former strain.

1.2.3.1 *Protection of young chicks against colonization*

Day-of-hatch chicks have no established gut flora and possess an immature mucosal immune system (Bar-Shira & Friedman, 2006). In the cecum, it is only after four to seven days post-hatch that an increase in cecal pro-inflammatory chemo- (such as interleukin-8 (IL-8)) and cytokine expression and heterophil numbers can be observed, upon exposure to feed and
microflora (Bar-Shira & Friedman, 2006). Hatchlings are also unprotected by adaptive immunity, which only starts to develop after a few days of life (Friedman et al., 2003). Nevertheless, colonization of chickens with C. jejuni during this critical period seems not to occur. Instead, maternally-derived antibodies generated against flagellin proteins, adhesins and other C. jejuni surface components are important in protecting young chickens from C. jejuni colonization during the first two weeks, the so-called lag-phase (Sahin et al., 2001, 2003; Shoaf-Sweeney et al., 2008; Zeng et al., 2009). Killing of C. jejuni by maternal antibodies happens in a complement-mediated, strain-specific way (Young et al., 2007). These antibodies confer enhanced protection against challenge with a homologous strain compared to a heterologous strain (Sahin et al., 2003), probably because they retard motility of a homologous, but not that of a heterologous strain, as shown in vitro (Shoaf-Sweeney et al., 2008). After the lag-phase, chickens show an increased susceptibility to colonization with C. jejuni which coincides with a loss of maternally-derived, circulating anti-Campylobacter IgY antibodies, suggesting that adaptive immunity is not critical in protecting broilers from colonization (Cawthraw & Newell, 2010). Interestingly, day-of-hatch broilers have been shown to be very susceptible to C. jejuni colonization. Susceptibility, however, diminished again over the first few days of life (Cawthraw & Newell, 2010; Conlan et al., 2011). Also, transmission of C. jejuni between co-housed birds is lower in day-old chicks compared to two-week-old birds (Conlan et al., 2011). This indicates that a lack of exposure of broiler flocks to C. jejuni and/or reduced transmission during the early stages of rearing may also contribute to the observed lag-phase.

Developing chicken embryos have increased expression levels of several avian β-defensins, a group of antimicrobial peptides important in innate and adaptive immune responses that might contribute to the observed protection toward C. jejuni infection in ovo and post-hatch (Meade et al., 2009a). For the β-defensin gallinacin-6, for instance, in vitro antibacterial activity against C. jejuni has been demonstrated (van Dijk et al., 2007).

1.2.3.2 Innate immune response

The chicken intestinal innate immune system is made up of several tissues, cells (such as epithelial cells, monocytes/macrophages, dendritic cells, natural killer cells and neutrophils) and germline-encoded molecules (such as chemo- and cytokines, antimicrobial peptides and nitric oxide) that can limit both commensal and pathogenic invading bacteria (Brisbin et al., 2008). Some in vitro studies with macrophages and epithelial cells, both primary and cultured, contributed to the insight into the chicken immune response toward C. jejuni infection. C.
jejuni has been shown to be adhesive to, invasive in and to stimulate inflammatory responses from these cells (Smith et al., 2005; Byrne et al., 2007; Larson et al., 2008; Van Deun et al., 2008b). Evidence of both in vitro uptake of C. jejuni by chicken peritoneal macrophages (Myszewski & Stern, 1991) and in vivo presence of C. jejuni within chicken epithelial cells and macrophages (Ruiz-Palacios et al., 1991) exists.

A crucial step in the host innate immune response to bacterial entrance in the GI tract is the activation of Toll-like receptors (TLRs), expressed on a variety of cells of the GI mucosa including macrophages and epithelial cells, the latter forming the first borderline defense against invading pathogens (He et al., 2006; Linde et al., 2008). TLRs are recognized by specific bacterial ligands and, once activated, promote the expression of effector molecules such as antimicrobial peptides, NO and inflammatory cytokines. Although knowledge on avian TLR biology is only starting to unravel, very recently several chicken TLRs have been implicated to play a role in C. jejuni recognition. The chicken TLR4/myeloid differentiation protein-2 (chTLR4/chMD-2) complex and cell-surface expressed chTLR2 recognize Campylobacter LOS and lipopeptides, respectively. Both receptors are potently activated by lysed Campylobacter bacteria. However, loss of bacterial cell wall integrity does not seem to play a critical role in TLR activation, because also live Campylobacter bacteria are able to elicit a marked inflammatory response in chickens (de Zoete et al., 2010). TLR5 specifically recognizes conserved regions of bacterial flagellins, thereby preventing intestinal pathology. C. jejuni, however, lacks these TLR5-recognition sites and is therefore unable to activate chTLR5, indicating that TLR5 signaling does not play a critical role in the chick immune response against C. jejuni (Guerry, 2007; de Zoete et al., 2010). Finally, TLR21, which is unique to avian, amphibian and fish species, enables recognition of unmethylated single-stranded microbial 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA motifs with a broad ligand specificity. C. jejuni CpG DNA is internalized through endocytosis and most likely interacts with chTLR21 intracellularly, similar to the interaction of CpG DNA with the functional homologue (TLR9) in mammals (de Zoete et al., 2010, Keestra et al., 2010).

Activation of chTLR2, chTLR4 and chTLR21 results in an innate immune response through myeloid differentiation primary response gene 88 (MyD88)-dependent activation of nuclear transcription factor kappaB (NF-κB) and subsequent production of inflammatory cytokines and chemokines (Brownlie et al., 2009; de Zoete et al., 2010; Keestra et al., 2010). Additionally, chTLR4 and chTLR21 ligands can induce the production of inducible nitric oxide synthase-mediated NO from chicken monocytes (He et al., 2006). In mammals, TLR-signaling also involves a TLR4-mediated MyD88-independent pathway associated with the
induction of late phase NF-κB and interferon (IFN)-inducible genes, such as IFN-β, involved in natural killer cell activation, and maturation of dendritic cells (Yamamoto et al., 2004). Chickens, however, lack this pathway and therefore have an aberrant response to C. jejuni LOS compared to mammalian species, rendering them much more resistant to the toxic effects of these TLR4 agonists. Although the TLR4-mediated MyD88-dependent pathway, leading to early phase activation of NF-κB, is intact, this explains in part the absence of pathological signs in chicks in response to infection with C. jejuni, despite cell adhesion and invasion (Keestra & van Putten, 2008; Shaughnessy et al., 2009; de Zoete et al., 2010).

Upon Campylobacter infection, primary chick kidney cells, primary chicken embryo intestinal cells and the avian macrophage cell line HD11 express NO and pro-inflammatory cyto- (IL-6 and IL-1β) and chemokines (chIL-8) (Larson et al., 2008; Li et al., 2008b). Production of NO by activated macrophages is important for their bactericidal activity (Linde et al., 2008). IL-1β and IL-6 are both major mediators of the innate immune system, while IL-6 is also involved in the immunological switch from innate to adaptive immunity (Smith et al., 2005). IL-1β is primarily produced by monocytes/macrophages and is involved in the inflammatory response of chickens against microbial products (such as LPS) by instructing epithelial cells and macrophages to produce chemokines (Bar-Shira & Friedman, 2006). The chicken orthologue of mammalian IL-8 (CXCLi1 and CXCLi2, but here referred to as chIL-8) (Kaiser et al., 1999; Smith et al., 2005) attracts heterophils and, unlike its mammalian counterpart, also monocytes to the site of infection (Martins-Green, 2001). It has been demonstrated that the N-terminus of chIL-8, where the chemotactic activity resides, is structurally homologous to that of monocyte chemotactic protein-1 (Borrmann et al., 2007). This human chemokine is chemotactic for monocytes, probably explaining the chemotactic movement of monocytes toward chIL-8. A marked chIL-8 response is induced in chicken LMH and primary intestinal cells upon inoculation with C. jejuni (Brisbin et al., 2008; Li et al., 20010). Finally, also in chicken embryo intestinal cells C. jejuni is capable of inducing a pro-inflammatory response (Borrmann et al., 2007; Smith et al., 2008).

Despite the lack of association of C. jejuni with chicken crypt epithelium in vivo, some recent reports demonstrate the initiation of a mild inflammatory response in chickens upon exposure to the bacterium. C. jejuni colonization in chicks after lag-phase is accompanied by infiltration of pro-inflammatory cells in mucosal tissues, although overt signs of cell invasion or pathology were not found (Larson et al., 2008; Smith et al., 2008). The number of circulating monocytes/macrophages is increased during colonization but, strikingly, heterophil numbers remain unaltered (Van Deun et al., 2008b; Meade et al., 2009b).
Expression of both TLR4 and TLR21 is readily increased in cecal tissues (Meade et al., 2009b; Shaughnessy et al., 2009) and is accompanied by a marked chIL-8 induction (Smith et al., 2008; Shaughnessy et al., 2009) and moderately induced IL-1β and IL-6 expression levels (Keestra & van Putten, 2008; Smith et al., 2008). Because the intestinal bacterial load in Campylobacter-colonized chicks does not lower, there clearly exist some mechanisms that are responsible for controlling this pro-inflammatory response (Smith et al., 2008). Expression levels of anti-inflammatory IL-10, IL-13 and transforming-growth factor β4 (TGF-β4) were not detected in cecum, ileum and spleen, and the signals modulating the pro-inflammatory response, resulting in sustained and unaffected C. jejuni colonization, are yet unknown (Smith et al., 2008; Shaughnessy et al., 2009). C. jejuni colonization in chicks significantly reduces expression levels of several antimicrobial peptide genes (Meade et al., 2009a). This downregulation might represent one mechanism whereby C. jejuni modulates the immune response, limiting the efficacy of these antimicrobial factors and enabling itself to persistently colonize its host at high levels. In a recent study by Shaughnessy et al. (2011) 270 genes were found to be significantly differentially expressed after 20 h in four-week-old chicks colonized with C. jejuni compared to C. jejuni-free chicks. These genes corresponded to the activation of several biological processes, including immune responses. Although differences in expression were only marginal, this response was hypothesized to point toward an innate T cell response in the ceca of chickens 20 h after inoculation with C. jejuni (Shaughnessy et al., 2011).

1.2.3.3 Adaptive immune response

The type of immune response generated against C. jejuni depends on the cytokine microenvironment induced by the chick innate defense cells. This in turn is determined by the interaction of TLRs and other pathogen recognition receptors expressed on these cells with their respective ligands. In chickens, not all of these receptors and cytokines are fully identified yet, making the switch from innate to adaptive immunity in this species not completely understood (Brisbin et al., 2008).

In chickens, intestinal antigens are capable of entering the bursa of Fabricius, the site of primary B cell development (Brisbin et al., 2008). Chickens have an incomplete antibody (IgM and IgY) response toward T cell-independent type 2 antigens, which activate B cells independently of T cells (Jeurissen et al., 1998). Because these antigens are usually of polysaccharide nature, an insufficient humoral response toward certain SACS of C. jejuni might contribute to the inability of the chicken immune system to clear this microorganism.
Despite the antigenic potential of *C. jejuni* LOS and its capsule (Oza *et al.*, 2002) and the marked immunogenicity of *C. jejuni* flagellin (Widders *et al.*, 1998). Moreover, an OMP extract of *C. jejuni* has been shown to cause apoptosis of chicken blood and spleen lymphocytes, probably promoting immune evasion of *C. jejuni* in the chick (Zhu *et al.*, 1999). An antibody response to *C. jejuni* might, however, contribute to protection against intestinal colonization of chickens, which show a significant increase in specific mucosal and circulating IgG (IgY) and IgA and circulating IgM antibody titres when colonized with *Campylobacter* (Cawthraw *et al.*, 1994; Widders *et al.*, 1998). In these studies, flagellin was shown to be the immunodominant antigen, which is rather peculiar due to the lack of functional TLR5-recognition sites in *C. jejuni* flagellin, permitting TLR5 evasion (Guerry, 2001; de Zoete *et al.*, 2010). Nevertheless, vaccinating chicks with a hybrid protein based on *C. jejuni* FlaA induced a specific response against this protein and reduced colonization in these birds (Khoury & Meinersmann, 1995). An antibody response specific for native flagellin was also induced in the serum of chickens immunized with purified *C. jejuni* flagellin. Serum and GI secretion antibodies specific for *C. jejuni* whole cells were, however, only induced when flagellin was complemented with killed *C. jejuni* whole cells, resulting in reduced cecal *C. jejuni* counts in these birds (Widders *et al.*, 1998). This might indicate that the epitopes of *C. jejuni* flagella are not accessible for these antibodies in intact bacteria and that possibly other antigens, not detected in this study, were responsible for the induction of anti-*C. jejuni* antibodies reducing the cecal bacterial load. Recent studies gave more insight into this enigma and identified additional immunogens of *C. jejuni* promoting the humoral immune response in chicks. Amongst others CfrA, CmeC, Cj0091 (belonging to a lipoprotein-encoding operon), the lipoproteins CjaA and CjaC (mediating amino acid transport), CadF and LOS were shown to be immunogenic and expressed during *in vivo* colonization (Shoaf-Sweeney *et al.*, 2008; Zeng *et al.*, 2009; Oakland *et al.*, 2011). Both the sera of young chicks free of *C. jejuni* and older birds colonized with the bacterium were reactive against recombinant CfrA, indicating that they are not only passed from the mature hen to the hatchling but are also induced during colonization of broilers after the lag-phase (Zeng *et al.*, 2009).

Intestinal epithelial cells might contribute to a mucosal IgA response by the GALT, located beneath the epithelial cell border in the lamina propria, in a T cell-dependent manner by producing IL-6 after contact with *C. jejuni* (Fagarasan, 2008). Secretory IgA is generally responsible for preventing sub-epithelial translocation of commensal bacteria by preventing their adhesion to epithelial cells or returning bacteria that already reached the basolateral site,
without eliciting an inflammatory response (Brisbin et al., 2008). Moreover, by its resistance to normal intestinal proteases, through dimerization on the surface of mucosal epithelial cells, IgA is ideally suited for host defenses at the mucosal surface of the GI tract (Phalipon et al., 2002). IgA might thus play an important role in limiting the mucosal immune response to \textit{C. jejuni} in chickens and redirecting it toward tolerance.

Most \textit{C. jejuni} strains possess genes encoding a cell death-promoting cytolethal distending toxin (CDT) of which the expression is induced in both the avian and human gut (Abuoun et al., 2005). During human infection with \textit{C. jejuni}, neutralizing antibodies against CDT are induced but not during colonization in chickens. It seems that production of this toxin in general is not important for chick colonization as opposed to its suspected role during pathogenesis in humans (Abuoun et al., 2005; Biswas et al., 2006).

As mentioned earlier, genetically distinct chicken lines may differ in their susceptibility toward cecal \textit{C. jejuni} colonization (Stern et al., 1990a). Further research in this area revealed insulin receptor signaling and metabolism process pathways to be key players of this differential response (Li et al., 2010). In a more resistant line, lymphocyte activation, lymphoid organ development functions and circadian rhythm were important in the cecal host defense upon \textit{C. jejuni} inoculation. In a more susceptible line, cell differentiation, communication and signaling pathways were important during host defense, with a marked upregulation in lipid, glucose and amino acid metabolism.

1.2.3.4 \textit{Chicken systemic immune response to C. jejuni}

Commensal bacteria in general do not colonize outside the GI tract, but strangely enough \textit{C. jejuni} can readily be found in various extraintestinal organs of broilers too. Up to seven days after oral and cloacal inoculation, the bacterium was found in the thymus, spleen, liver/gallbladder and bursa of Fabricius (Cox et al., 2005; Van Deun et al., 2008b; Meade et al., 2009b). In a study with two-week-old chicks that were inoculated with \textit{C. jejuni} at day-of hatch, high bacterial numbers (> 5 log cfu/g) were isolated from spleen and liver of most of the birds (Lamb-Rosteski et al., 2008). In addition, \textit{C. jejuni} was isolated from the reproductive tract and ovarian follicles of laying hens (Cox et al., 2009). The dissemination of \textit{C. jejuni} to other organs seems to be correlated with the invasive potential in primary cecal epithelial cells of chicks (Van Deun et al., 2008b), suggesting that \textit{C. jejuni} translocates the epithelial barrier transcellularly (through the chicken crypt epithelium) rather than paracellularly (between cells).
This frequently observed systemic colonization indicates that C. jejuni, despite the induction of secretory IgA by the GALT, is capable of breaching the gut epithelial barrier. As in the GI tract, this happens without developing pathology or inducing excessive inflammation, although chicks can mount an adaptive T cell response to C. jejuni when it reaches and colonizes the liver (Jennings et al., 2011). In colonized flocks, almost all birds carry C. jejuni in their ceca but significantly less birds harbour the bacteria in their liver tissues (Jennings et al., 2011). Whether host-specific differences decide over C. jejuni dissemination, or a T cell response is responsible for the eradication of C. jejuni from the host liver in some animals, is not known. In any case, C. jejuni-specific antibody responses are apparently not capable of clearing the bacterium from the chicken gut, but nevertheless do indicate that there indeed must have been a preceding close interaction between C. jejuni and the host epithelial cells.

The two chicken lines used in the study of Li et al. (2010) also differed in their systemic response to C. jejuni (Li et al., 2011). In the spleen, a secondary lymphoid organ of the avian immune system important for lymphocyte activation, proliferation and differentiation, the response to C. jejuni in the more resistant line was characterized, as in the cecum, by lymphocyte activation and differentiation. In addition, splenic host genes for humoral responses and Ig heavy and light chain were upregulated. These responses initiate adaptive immune responses to C. jejuni and are probably responsible for an increased genetic resistance to systemic C. jejuni colonization. In the susceptible line, genes for regulation of erythocyte differentiation, hematopoiesis and RNA biosynthesis processes were downregulated. This study also revealed distinct innate defense mechanisms against C. jejuni by the two chicken lines. Apoptosis and cytochrome c release from mitochondria was associated with increased resistance against C. jejuni colonization. Probably, these events induce increased apoptosis of infected host cells, thereby destroying the habitat of the bacteria and contributing to the increased resistance to splenic colonization with C. jejuni.

1.2.4 Interaction with the host microbiota

Little is known currently about the effect of the natural avian gut microbiota on the level of C. jejuni colonization. In general, host microbiota imposes a colonization barrier for intruding pathogens by competing for nutrients and host receptors. Their composition, however, can alter the outcome of invading enteric bacteria (by e.g. altering the virulence properties of these bacteria), resulting in either clearance or colonization (Keeney & Finlay, 2011). Although it has been suggested that the colonization pattern of C. jejuni in chicks is mainly
determined by the chicken host and not by the host microbiota (Ridley et al., 2008b), also the composition of the latter might contribute to the observed colonization pattern. Changes in C. jejuni loads in the commercial turkey intestine seemed to correlate to, but were not dependent on, two acute transitions in the cecal microbiota composition during the turkey development phases (Scupham, 2009). With an approach called antibiotic dissection, day-old turkey poulters were inoculated with cecal contents of Campylobacter-free adult turkeys after which the microbial communities in these poulters were modified by different antibiotic treatments. Molecular examination of the constituents of these communities detected that a subtype I of Megamonas hypermegale correlated with decreased colonization ability of C. jejuni, while a virginiamycin-derived cecal microbiota seemed to be correlated with enhanced colonization ability (Scupham et al., 2010). These results indicate that C. jejuni may respond to the presence of specific subsets of the avian gut microbiota. It has, however, to be examined if the effect of these gut microbiota alterations on C. jejuni in turkeys also applies to chicks.

1.2.5 Hypothetical mechanism of the interaction between C. jejuni and the chicken gut

The interaction of C. jejuni with its chicken host is very complex, evidenced by the extensive interplay between several key mediators important for successful and persistent colonization of the chicken GI tract. Clearly, this dual interaction is influenced by both the C. jejuni strain and the chicken line involved. Despite the lack of a developed pathology, a pro-inflammatory response is developed in the chicken intestinal mucosa during asymptomatic colonization with C. jejuni, suggesting a significant interaction between the bacteria and the epithelial cell layer of the chicken gut.

Upon entering the chicken GI tract, C. jejuni moves toward the intestinal epithelial border, mediated by chemotaxis. C. jejuni is attracted by intestinal mucins, as well as several amino acids, carbohydrates and salts of organic acids, while the chemoattractive properties of L-fucose are controversial (Vegge et al., 2009). C. jejuni responds to these chemicals via methyl-accepting chemotaxis proteins (Vegge et al., 2009). For moving toward the most favourable conditions for growth, C. jejuni needs intact flagella and it seems that especially flaA, flgK, cj1324 and maf5 are crucial for colonizing the chicken gut (see 1.2.2.3). Upon reaching the chicken gut epithelial boarder it is believed that C. jejuni adheres to the epithelial cells, mediated by intact flagella and surface-exposed proteins. In particular CadF and FlpA were identified as important adhesins, while the potential contribution of CapA is less clear (see 1.2.2.7). Also several surface-accessible carbohydrate structures of C. jejuni, such as
LOS and an intact capsule (see 1.2.2.4) are involved in adhesion, with in particular \textit{kpsM} and \textit{pglH} being important for colonization of the chicken intestinal tract. Adhesion of \textit{C. jejuni} to gut epithelial cells may be followed by marginal invasion in these cells. Upon exposure to chicken mucus, the flagellar apparatus increases the secretion of Cia proteins. Also \textit{C. jejuni} LOS is important for epithelial cell invasion and sialylation of the LOS outer core enhances this trait (Louwen \textit{et al.}, 2008; Habib \textit{et al.}, 2009). \textit{C. jejuni} is not able to survive for long periods in primary chicken cecal epithelial cells (Van Deun \textit{et al.}, 2008b). Therefore, intracellular replication in these cells is probably not important for persistent \textit{in vivo} colonization. Rather, invasion of cecal crypt epithelial cells would be followed by evading these cells allowing \textit{C. jejuni} to replicate in the mucus, which seems to provide all necessary nutrients for optimal growth, and re-invasion to escape mucosal clearance (Van Deun \textit{et al.}, 2008b). Strangely, the invasion capacity of \textit{C. jejuni} in primary chicken cecal epithelial cells \textit{in vitro} is not correlated with \textit{in vivo} gut colonization (Van Deun \textit{et al.}, 2008b). Therefore, the genuine contribution of epithelial cell invasion during cecal colonization of chicks with \textit{C. jejuni} is not clearly definable and can only be speculated on.

\textit{C. jejuni} entrance in the avian GI tract induces an early production of chIL-8 by intestinal epithelial cells, followed by macrophage recruitment and production of pro-inflammatory cytokines. This is, however, not accompanied by the recruitment of heterophils (the avian equivalent of mammalian neutrophils) to the site of infection. In a later stage, a specific mucosal IgA response is mounted against \textit{C. jejuni}, but this induction is not capable of clearing the bacterium from the gut. This humoral response is moreover not able to prevent \textit{C. jejuni} from further interacting with and translocating across the gut epithelium and to disseminate systemically. Also the specific T cell response that is triggered upon \textit{C. jejuni} entrance in the extra-intestinal organs does not result in clearance from these tissues, or pathology. \textit{C. jejuni} is thus capable of evading the inefficient host immune response and the chicken host might be involved in maintaining homeostasis during persistent colonization (see 1.2.6).

In \textbf{figure 1}, a schematic overview is given of a simplified hypothetical mechanism involved in the interaction of \textit{C. jejuni} with the chicken gut, after lag-phase, leading to successful and persistent colonization of the GI tract, without developing pathology.
Figure 1 Intestinal *Campylobacter jejuni*-chicken crosstalk leading to persistent colonization. (A) Colonization mechanism. Upon entering the chicken gastro-intestinal (GI) tract, *C. jejuni* is attracted to the mucus layer by chemoattractants sensed by methyl-accepting chemotaxis proteins (MCP). Intact flagella are critical for this movement and allow *C. jejuni* to further migrate toward the epithelial barrier where several of its surface-accessible carbohydrate structures (SACS) modulate adhesion to and invasion of epithelial cells. Also mucus modulates invasion, by inducing the secretion of *Campylobacter* invasion antigen B (CiaB). An invasion-evasion cycle followed by replication in the mucus is probably responsible for the high numbers at which *C. jejuni* colonizes the GI tract. The chick immediately responds to *C. jejuni* entry by chIL-8 production from epithelial cells and recruitment of macrophages. Several other mechanisms such as stress responses are further essential for both initial and persistent colonization of *C. jejuni* in the chicken GI tract. Long-term colonization is followed by the induction of specific mucosal immunoglobulin A (IgA), but *C. jejuni* translocation across the epithelial barrier toward the lamina propria, possibly by a transcellular route, is not prevented. (B) Intracellular inflammatory response. Expression levels of chicken Toll-like receptor 4 (chTLR4) and 21 (chTLR21) are increased in both epithelial cells and macrophages, which produce nuclear factor-κB (NF-κB)-mediated cytokines (IL-1β, IL-6, chIL-8) after binding their specific ligands, by triggering the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway. *C. jejuni* lipooligosaccharide (LOS) is sensed by the surface-exposed complex chTLR4/ch myeloid differentiation protein-2 (MD-2), while *C. jejuni* 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) DNA is endocytosed and recognized by intracellular TLR21. This controlled inflammatory response is neither followed by pathological signs, nor clearance of the bacterium from the GI tract.
1.2.6 Commensal *C. jejuni* colonization in chicks: immunological tolerance?

In mammals, commensal infections are characterized by the absence of a neutrophil infiltrate or a classical inflammation as seen during pathogenic infection (MacPherson & Uhr, 2004), indicating that the interaction between *C. jejuni* and its chicken host is indeed of commensal nature. Intestinal homeostasis during commensal colonization requires that a pro-inflammatory response is rapidly controlled. In mammals, not much is known about the host regulatory mechanisms that contribute to tolerance without reducing bacterial numbers, but, restricting the bacteria to the lumen (so they cannot reach the epithelial cells and the immune system) and inducing an anti-inflammatory response are believed to induce a state of “immunological ignorance” (Ivanov & Littman, 2011). Due to a lack of knowledge about the interaction between *C. jejuni* and the chicken immune system, it remains unclear how homeostasis is maintained in chickens colonized with *C. jejuni*. An apparent induction of a mild intestinal pro-inflammatory response, the inability to demonstrate upregulation of anti-inflammatory cytokines, occasional invasion of cecal crypt epithelial cells and regular dissemination to extra-intestinal organs upon *C. jejuni* colonization of the chicken host, suggests that their interaction is not a tale of ignorance but rather a cohort of active processes, exerted by the two partners, resulting in “immunological tolerance”. *C. jejuni* itself might escape or alter the inflammatory response by, for instance, down-regulating antimicrobial peptide gene expression in the chicken gut, but other potential mechanism(s) or bacterial factor(s) of *C. jejuni* involved in immune evasion are currently not known. Alternatively, the chicken host might support tolerance to maintain homeostasis during persistent, asymptomatic colonization (Pédron & Sansonetti, 2004).

First of all, the differential composition of the chicken intestinal mucus layer, compared to its human counterpart, probably plays an important role in promoting homeostasis during *C. jejuni* colonization. Chicken intestinal mucins have been shown to reduce the adhesive and especially the invasive capacity of *C. jejuni* in human primary and cultured intestinal epithelial cells (Byrne *et al*., 2007; Alemka *et al*., 2010). In contrast, human-derived mucus promotes adhesion and entrance (Byrne *et al*., 2007). Moreover, MUC2, the most abundantly secreted mucin in the human intestine, is a major chemoattractant for *C. jejuni* and induces the expression of several colonization- and virulence-associated genes (Tu *et al*., 2008). To date, no such properties have been assigned to chicken mucins. Host intestinal mucins can be either secreted or expressed at the apical surface of the (cecal) mucosal epithelial cells and are readily found to be coated with fucosylated glycans in terminal positions (Stahl *et al*., 2011).
Although the chemotactic properties of L-fucose were not validated by Vegge et al. (2009), it is believed that *C. jejuni* is attracted to, and binds with both mucin and L-fucose. Presence of the latter at certain concentrations might moreover increase *C. jejuni* flaA promoter activity (Allen et al., 2001). Therefore, fucosylated glycans may function as adherence factors for *C. jejuni*. In addition, although it was believed until now that *C. jejuni* is an asaccharolytic organism, very recent evidence indicates that some strains are able to utilize L-fucose as a substrate for growth (Stahl et al., 2011). Thus, chemotaxis toward, adhesion to and subsequent utilization of L-fucose by *C. jejuni* strains possessing a functional L-fucose uptake and metabolism pathway provides them with a competitive advantage. This seems, however, to be only the case during pathogenic (human), but not during commensal (chick) colonization (Stahl et al., 2011), were chemotaxis toward amino acids is believed to be less effective due to the higher body temperature (Baserisalehi & Bahador, 2011). Probably, next to decreasing the intestinal barrier permeability to *C. jejuni*, the highly sulfated fucosylated O-glycan mucin structures found in chickens decrease the accessibility of, and thus the responsiveness of *C. jejuni* to L-fucose. Indeed, upon feeding young chicks with an excess of free L-fucose also here a competitive colonization advantage was observed for wild-type *C. jejuni* over a mutant lacking a functional fucose permease gene, important for L-fucose transport into the bacterial cell (Stahl et al., 2011). Thus, a high degree of L-fucose masking through increased sulfation might give further explanation to the lack of association of *C. jejuni* with the chicken crypt epithelium in vivo. To conclude, there is increasing evidence that the composition of the chicken mucus layer is involved in the hindered contact between *C. jejuni* with the chicken intestinal epithelial surface. Indeed, *C. jejuni* is not closely associated with chicken crypt epithelium in vivo but rather resides in the mucus within the lumen of the crypts (Beery et al., 1988). However, the effect of chicken mucus on *C. jejuni* invasion in primary chicken epithelial cells has not yet been examined. Moreover, as the bacterium can be frequently detected in extra-intestinal organs of chicks, the mucus layer is not likely to be an efficient barrier to prevent close interaction with *C. jejuni* and the intestinal epithelial lining. In contrast, it seems that it indirectly promotes *C. jejuni* invasion through the secretion of Cia proteins (Biswa et al., 2007). Further research will therefore have to reveal the genuine contribution of the mucus layer to GI and systemic colonization of *C. jejuni* in chicks.

Also the adaptive immune system of the chick might participate in the tolerogenic response to *C. jejuni*. Upon intestinal colonization, specific IgA against *C. jejuni* is induced. IgA is believed to induce the modulation of epitope expression by bacteria and to reduce intestinal pro-inflammatory signaling (Peterson et al., 2007). This indicates that the induction of IgA
could lead to immune evasion, but whether the induction of IgA in chickens colonized with *C. jejuni* might be responsible for the non-inflammatory *C. jejuni*-chicken gut relationship is not clear.

Next, murine intestinal epithelial cells are tolerized to LPS early after birth by exposure to exogenous LPS, facilitating microbial colonization and the establishment of a stable intestinal host-microbe homeostasis (MacPherson & Uhr, 2004). Whether in chickens LOS tolerance in the gut is involved in a tolerance-oriented integrated mucosal immune system, allowing commensal colonization of *C. jejuni*, is not clear.

Finally, chickens have an aberrant response to *C. jejuni* LOS and flagellin, due to the absence of a late phase NF-κB response and TLR5 recognition sites, respectively. Only the first is likely to contribute to the differential *C. jejuni* response in humans and chicks because *C. jejuni* escapes TLR5 recognition in humans too (de Zoete et al., 2010). Next to these responses, colonized chickens might further induce tolerance by expressing factors that blunt *C. jejuni* components which could induce inflammation (MacPherson & Uhr, 2004). However, potential candidates have not yet been identified.

### 1.2.7 Conclusion

Upon entering the chicken GI tract, *C. jejuni* establishes a complex interaction with its host, resulting in persistent high-level cecal colonization. This inefficient, controlled inflammatory response is not capable of clearing *C. jejuni* from the chicken gut and many processes might be involved in redirecting the response toward tolerance. The underlying mechanisms of the crosstalk between *C. jejuni* and chicks are just now starting to be unravelled and further research is warranted. Especially the mechanisms allowing this bacterium to persistently evade the immune response should deserve full attention. After all, a better understanding of the chick immune response upon *C. jejuni* entrance, as well as further elucidation of the colonization mechanism of the bacterium in this host might promote the development of effective control measures to clear this human pathogen from poultry lines. For this purpose, it might be of particular interest to identify chicken factors, if any, involved in blunting *C. jejuni* virulence factors, while *C. jejuni* colonization factors identified to date might hold promise for effective subunit vaccines. Moreover, the differential disease outcome in chicks and humans upon exposure to *C. jejuni* needs to be explained. Could it be due to the differences in mucin composition, TLR signaling, effect of CDT or humoral responses in these hosts, or are there other, yet to define, mechanisms that determine the commensal or
pathogenic nature of *C. jejuni*. Answering these questions could explain why and how a single bacterium is capable of causing severe inflammatory disease in one host while being (seemingly?) completely harmless in another.

Besides genes which are probably necessary during colonization of the GI tract in a wide range of animal species in general, it seems that *C. jejuni* needs a distinct set of gene products for optimal adaptation to the chicken intestinal environment, resulting in high-level cecal colonization. However, the mechanisms by which these factors interplay to form the basis behind the complex interaction of *C. jejuni* with its avian host remain largely unclear. The incomplete understanding of the dual interaction between *C. jejuni* and the chick immune system is at least in part responsible for the lack of effective control measures (see 1.3).

### 1.3 **CAMPYLOBACTER CONTROL IN POULTRY**

In the past few years, several quantitative risk assessments for *Campylobacter* in poultry meat have been developed as a guidance tool to control the presence of this zoonotic pathogen throughout the poultry meat production chain (Nauta *et al*., 2009). Although there is considerable variation between countries in the approach of these models, all risk assessments conclude that aiming to reduce the *Campylobacter* levels on broiler carcasses after evisceration is the most effective intervention measure, rather than reducing its prevalence. Besides reducing external surface contamination of broiler carcasses from *Campylobacter*-colonized flocks directly, by physical or chemical means (Rosenquist *et al*., 2006; Boysen & Rosenquist, 2009), reduced *Campylobacter* numbers on carcasses can also be obtained indirectly. On-farm intervention measures aim to prevent *Campylobacter* introduction and transmission in poultry flocks or to reduce intestinal *Campylobacter* counts in colonized animals leading to reduced contamination levels of the carcasses of these animals after processing. Moreover, because the intestine of living poultry is the only amplification site for *Campylobacter* throughout the entire food chain, reducing the cecal *Campylobacter* load in poultry during primary production is expected to significantly reduce the incidence of human campylobacteriosis (Lin, 2009).

In Denmark, a quantitative microbial risk assessment of human campylobacteriosis associated with thermotolerant *Campylobacter* spp. in broiler chickens was developed. The simulations showed that reducing the number of *Campylobacter* bacteria on chicken carcasses by 2 logs
causes a 30-fold reduction in the incidence of campylobacteriosis in humans (Rosenquist et al., 2003). Bacterial counts in cecal contents of Campylobacter-colonized broiler flocks should be reduced by roughly one log more (i.e. 3 logs) to obtain a similar reduction in the number of human campylobacteriosis cases (Reich et al., 2008). A Belgian risk assessment showed that the incidence in Belgium would be reduced by 48%, 85% and 96% when respectively a one log, two log or three log reduction of the Campylobacter contamination on carcasses would be achieved (Messens et al., 2007).

Theoretically, controlling Campylobacter colonization in poultry on-farm may be achieved in a number of different ways, including hygienic and biosecurity measures (1.3.1), water treatment (1.3.2), supplementing plant-derived additives to the feed (1.3.3), bacteriophage application (1.3.4), vaccination (1.3.5), passive immunization (1.3.6) and application of competitive exclusion microflora, probiotics and prebiotics (1.3.7) or bacteriocins (1.3.8). It is important to differentiate between prevention and colonization-reducing measures, which intervene at a different stage of the colonization process. Preventive measures, summarized in table 2, aim at reducing the probability of birds to become colonized with Campylobacter, while colonization-reducing measures, presented in table 3, strive for a reduced cecal Campylobacter load in colonized birds prior to slaughter, thereby reducing surface contamination of the carcasses. Moreover, also by improving health and welfare of the animals colonization might be reduced (Bull et al., 2008). Finally, genetic selection could also contribute in combating Campylobacter colonization in poultry (Kapperud et al., 1993), when poultry lines with improved overall immunological responsiveness, being more resistant to colonization with this pathogen, are developed (Swaggerty et al., 2009). Some antibiotics efficiently reduce C. jejuni counts in the broiler chick GI tract (Farnell et al., 2005), but their use is controversial due to concerns on development of antibiotic resistance in C. jejuni, which may compromise treatment of human campylobacteriosis (Dibner & Richards, 2005; Zhu et al., 2006).
Table 1 Preventive on-farm control measures to reduce *Campylobacter* prevalence and transmission in poultry flocks.

<table>
<thead>
<tr>
<th>Intervention measure</th>
<th>Intervening stage in colonization process</th>
<th>Points of attention</th>
<th>References</th>
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<tbody>
<tr>
<td><em>Hygiene and biosecurity farming practices</em></td>
<td>Reduced prevalence of <em>Campylobacter</em>-colonized flocks</td>
<td>Need to be properly applied; not enough as a single intervention measure due to constant contamination pressure</td>
<td>van de Giessen et al., 1998 Gibbens et al., 2001 Hald et al., 2007 Rosenquist et al., 2009</td>
</tr>
<tr>
<td><em>Drinking water treatment</em></td>
<td>Reduced transmission as well as reduced crop and pre-chill carcass contamination by adding organic acids</td>
<td>Only a limited effect on <em>Campylobacter</em> prevalence in broiler chickens</td>
<td>Chaveerach et al., 2002 Chaveerach et al., 2004b Ellis-Iversen et al., 2009 Stern et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Reduced risk for <em>Campylobacter</em> colonization when chlorinating drinking water</td>
<td>No reduced <em>Campylobacter</em> prevalence observed under commercial settings</td>
<td></td>
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<tr>
<td><em>Feed additives</em></td>
<td>Reduced crop contamination and reduced probability of chickens to become colonized when organic acids are supplemented to the feed</td>
<td>Contradictory observations</td>
<td>Heres et al., 2004 Van Deun et al., 2008a Solis de los Santos et al., 2008 Skanseng et al., 2010 van Gerwe et al., 2010</td>
</tr>
<tr>
<td><em>Vaccination</em></td>
<td>Increased resistance to colonization with <em>Campylobacter</em> upon treating with killed whole cells and subunit vaccines</td>
<td>Incomplete understanding of the chicken immune system hampers development of an effective vaccine; genomic instability of <em>C. jejuni</em>?</td>
<td>Widders et al., 1996 Rice et al., 1997 Widders et al., 1998 Khoury &amp; Meinersmann, 1995 Wyszynska et al., 2004 Buckley et al., 2010</td>
</tr>
<tr>
<td></td>
<td>No <em>Campylobacter</em> could be recovered from ileum with subunit (Omp18/CjaD) <em>Salmonella</em>-vectored vaccine</td>
<td>Cecal <em>Campylobacter</em> counts were not determined</td>
<td>Layton et al. 2010</td>
</tr>
<tr>
<td><em>Passive immunization</em></td>
<td>Substantial reduction (&gt;99%) in fecal <em>C. jejuni</em> counts upon treating with Immunoglobulin preparations</td>
<td>Optimization and experimental trials in industrial or farm settings are needed to prove efficacy</td>
<td>Stern et al., 1990b Tsubokura et al., 1997</td>
</tr>
<tr>
<td><em>Competitive exclusion microflora</em></td>
<td>Increased resistance to <em>Campylobacter</em> colonization with feeding animals (un-)defined microbiota</td>
<td>Contradictory observations</td>
<td>Line et al., 1998 Morishita et al., 1997 Schoeni &amp; Wong, 1994 Svetoch &amp; Stern, 2010</td>
</tr>
<tr>
<td></td>
<td>Competitive exclusion of human pathogenic <em>C. jejuni</em> strains by characterized hyper-colonizing <em>C. jejuni</em> strains</td>
<td>Needs further research regarding applicability under commercial settings</td>
<td>Chen &amp; Stern, 2001 Calderon-Gomez et al., 2009</td>
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</table>
**Prebiotics**
Reduction in cecal *Campylobacter* load in animals fed mannanoligosaccharide or xylanase
Limited reducing potential
Baurhoo *et al*., 2009; Fernandez *et al*., 2000

**Antibiotic dissection**
A subtype I of *Megamonas hypermegale* specific for a *C. jejuni*-suppressive treatment
In vivo experiments are needed to prove *C. jejuni* exclusion in poultry
Scupham *et al*., 2010

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**Table 2** On-farm intervention measures to reduce the cecal *Campylobacter* load of already-colonized broiler chickens

<table>
<thead>
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<th>Effect on cecal <em>Campylobacter</em> numbers</th>
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<td>Hilmarsson <em>et al</em>., (2006) Metcalf <em>et al</em>., (2011) Byrd <em>et al</em>., 2001</td>
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<tr>
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<td>Reduced cecal <em>Campylobacter</em> load in broilers fed organic acids</td>
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<td>Hilmarsson <em>et al</em>., (2006) Solis de los Santos <em>et al</em>., (2010)</td>
</tr>
<tr>
<td><strong>Passive immunization</strong></td>
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<td>Optimization and experimental trials in industrial or farm settings are needed to prove efficacy</td>
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<td><strong>Bacteriocins</strong></td>
<td>Supplementation of BCN 760 in drinking water capable of complete eradicating <em>C. jejuni</em> in 90% of the cases</td>
<td>Safety? Long-term efficacy?</td>
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</tbody>
</table>
1.3.1 Hygienic and biosecurity farming practices

Good hygienic farming practices constitute a strategy aimed to prevent the introduction of *Campylobacter* into a flock by a combination of hygiene and biosecurity measures. A Belgian quantitative microbial risk assessment showed that the incidence of human campylobacteriosis in Belgium would be reduced by 32%, 53% and 77% when the *Campylobacter* flock prevalence is reduced by 25%, 50% or 75% respectively (Messens *et al.*, 2007). Application of specific hygienic measures during the rearing period, such as washing hands before entering the chicken house, the use of separate boots for each broiler house, footbath disinfection when entering a broiler house and a high standard of cleaning and disinfection of the drinking water equipment may significantly reduce the risk of *Campylobacter*-colonized broiler flocks (van de Giessen *et al.*, 1996; Evans & Sayers, 2000).

After introduction of hygienic and biosecurity measures, including the control of rodents and insects, in two Dutch broiler farms, the ratio of *Campylobacter*-colonized flocks compared to the total amount of flocks decreased from 12/18 at one farm and 4/4 at the second farm to 2/9 and 5/12, respectively (van de Giessen *et al.*, 1998). In the UK, the implementation of an intervention trial, based on a standard hygiene protocol for personnel and proper disinfection of the broiler house prior to stocking, reduced the prevalence of *Campylobacter* infection in the broiler population from 80% to < 40% (Gibbens *et al.*, 2001). It has been demonstrated that the prevalence of broiler flocks colonized with *Campylobacter* can be reduced from 51.4% to 15.4% by placing fly screens in broiler houses (Hald *et al.*, 2007). In Denmark, biosecurity strategies to control *Campylobacter* on-farm were intensified in 2003 leading to a decrease of *Campylobacter*-colonized flocks from 43% in 2002 to 27% in 2007 (Rosenquist *et al.*, 2009). These findings suggest that proper application of biosecurity measures can lead to reduced *Campylobacter* colonization in poultry. However, because broiler chickens are under a constant contamination pressure, biosecurity measures alone will not be sufficient to solve the problem.

1.3.2 Drinking water treatment

By treating the drinking water for poultry flocks, the risk of the animals to become infected might be reduced, probably through a reduction in bacterial numbers both in the drinking water and the crop. In this way, *Campylobacter* is less likely to reach the ceca and transmission throughout the flock might be reduced or prevented.
In vitro studies have demonstrated that organic acids have a strong bactericidal effect on Campylobacter spp. and addition of these acids to the drinking water on poultry farms could prevent transmission through broiler flocks (Chaveerach et al., 2002; Chaveerach et al., 2004b). Preventive addition of monocaprin, the mono-acylglycerol of capric acid (Thormar et al., 2006), to the drinking water resulted in a reduced C. jejuni count on cloacal swabs of approximately 2 logs, two days after experimentally inoculated chicks were co-housed with C. jejuni-free birds (Hilmarsson et al., 2006). Extending the treatment period, however, resulted in a reduced effectiveness of the treatment at days 5 and 10 after birds were co-housed. Moreover, on day 13 no significant reduction was observed in cecal C. jejuni counts of birds receiving monocaprin, indicating that this treatment did not prevent C. jejuni spread from artificially infected to non-infected birds. Chlorinating the drinking water is helpful as it reduces the risk for Campylobacter colonization (Ellis-Iversen et al., 2009). Chlorination of flock drinking water (with 2-5 ppm chlorine) under commercial production practices in the US in 2002 did, however, not result in a reduced Campylobacter prevalence in the birds receiving treated water (Stern et al., 2002).

Alternatively, addition of monocaprin to drinking water from the last three days before slaughter resulted in a reduced C. jejuni count of approximately log 1.85 on cloacal swabs of naturally infected birds, compared to control birds (Hilmarsson et al., 2006). Addition of 0.44% (vol/vol) lactic acid in the drinking water during pre-slaughter feed withdrawal reduced the incidence of both crop (by 22.8%) and pre-chill carcasses (by 14.7%) contamination with Campylobacter compared to control groups (Byrd et al., 2001). Finally, Metcalf et al. (2011) observed a 3 log reduction in cecal Campylobacter counts of 14-d-old chicks receiving drinking water supplemented with 0.175% (wt/vol) caprylic acid. However, this decrease was not consistent and moreover absent when applying different caprylic acid doses.

1.3.3 Feed additives

Changes in the composition of the feed can promote gastro-intestinal health and thus contribute to the control of Campylobacter in poultry. Plant-derived antimicrobial feed additives can be administered from day-of-hatch to prevent broiler chickens to become colonized and to reduce Campylobacter transmission throughout the flock. Also in this application, the observed effect is largely due to the anti-Campylobacter effect in the crop of the animals.
Next to their application in drinking water, organic acids might also be used as feed additives to reduce *Campylobacter* prevalence in poultry. However, *in vivo* trials demonstrated only a limited effect of feed acidification on *C. jejuni* prevalence in broiler flocks. At most it could delay the onset of colonization (Heres et al., 2004; Line & Bailey, 2006). Broilers that were fed fermented liquid feed, i.e. a moistened feed with a high number of lactobacilli, a high concentration of lactic/acetic acid and a pH of 4, were less likely to shed *Campylobacter* after oral infection (Heres et al., 2003). However, at the end of the trial no significantly different *C. jejuni* counts in the ceca could be observed compared to chickens on a standard feed. The higher level of lactic acid in combination with a low pH in the crop was suggested to reduce the probability for *Campylobacter* to reach the ceca. In a later experiment, individually housed chickens that were fed acidified feed were found to be less susceptible to *Campylobacter* infection compared to control birds, as less chickens became colonized at equal inoculation doses (Heres et al., 2004). Also caprylic acid leads to reduced colonization in 10-day-old chicks when given preventively (Solis de los Santos et al., 2008). In contrast, addition of butyrate to the feed was not able to reduce cecal *Campylobacter* colonization in a seeder model using two-week-old broilers (Van Deun et al., 2008a). Skanseng et al. (2010) found little effect when supplementing only formic acid to the feed, but a combination of 2% formic acid with 0.1% sorbate prevented *C. jejuni* colonization in chicks. Finally, it was demonstrated that the addition of a medium-chain fatty acid mixture to the feed at 1% reduces the probability of broilers becoming colonized (van Gerwe et al., 2010). Administration of large molecules that interfere with *Campylobacter* adhesion to the host cell is successful *in vitro* but suffers from premature metabolic breakdown in the broiler chicken GI tract (Wittschier et al., 2007). Finally, cecal colonization of birds receiving plant-protein-based feed was significantly lower compared to birds receiving animal-protein-based feed or a combination of plant- and animal-protein sources (Udayamputhoor et al., 2003).

Alternatively, colonized broiler chickens might be fed pulse doses of the additives for a certain period, just before slaughter, aiming at reducing the cecal *Campylobacter* load and reducing carcass contamination after slaughter. In this application one aims to reduce the *Campylobacter* numbers in the ceca of already colonized birds. To reach the cecum efficiently, additives are often coated on/encapsulated in carrier material that will prevent premature degradation along the GI tract and assure efficient release of the active compound into the gut (Van Immerseel et al., 2004). Addition of monocaprin to the feed from the last three days before slaughter, resulted in a reduced *C. jejuni* count on cloacal swabs of naturally infected birds (Hilmarsson et al., 2006). In addition, Solis de los Santos et al. (2010) observed
a considerable reduction (of several logs) in cecal *Campylobacter* numbers when caprylic acid was given from three days before slaughter, in already colonized market-aged broilers. This reduction was strikingly not accompanied by an altered cecal microbial population.

As the available *in vivo* results are limited and moreover contradictory, it cannot be univocally be determined what the contribution of feed additives will be to control cecal *Campylobacter* colonization. Preventive supplementation from day-of-hatch, rather than aiming for reduced cecal *Campylobacter* numbers in already colonized birds, seems most promising.

1.3.4 Bacteriophage application

Bacteriophage application to reduce cecal *Campylobacter* colonization in poultry is promising (Carrillo *et al.*, 2005; Wagenaar *et al.*, 2005). Results indicate an immediate drop in *C. jejuni* numbers of approximately three logs in already-colonized chicken ceca (Wagenaar *et al.*, 2005). After five days, however, bacterial counts stabilized at a level one log lower compared to control birds, an effect also observed when phages were given prophylactically. Also El-Shibiny *et al.* (2009) observed an immediate (after two days) two-log cfu/g reduction in cecal *C. jejuni* and *C. coli* levels. Despite the fact that *Campylobacter*, after a sudden drop, seems to re-establish itself to nearly its original counts, results do indicate that bacteriophages can possibly be successfully applied in broilers to reduce the cecal bacterial load just before slaughter. Further research in this area showed that administering phages in the feed is more efficient than oral gavage (Carvalho *et al.*, 2010). This study revealed an initial drop, already after two days, of approximately two logs in the numbers of *C. jejuni* in the fecal material of infected one-week-old birds using a phage cocktail. Moreover, *C. jejuni* did not regain its original counts throughout the experimental period, which was ended seven days after phage administration had started.

Although the use of phage products in broilers seems to be a promising way to reduce cecal colonization with *C. jejuni*, questions regarding both immediate and long-term efficacy, consumer safety and application methods arise (Hagens & Loessner, 2010). Safety concerns should not be a main obstacle as phages are highly specific and can only infect a limited range of host bacteria. Moreover, their oral consumption, even at very high levels, is believed to be completely harmless to humans. Answers concerning the efficacy seem to be more complex, especially if long-term efficacy of the phage product has to be ensured. In the study of El-Shibiny *et al.* (2009) it was shown that 2% of the *Campylobacter* population exposed to virulent phages in the chicken, developed phage-resistance. These resistant types remained a
minor component of the population. Carvalho et al. (2010) isolated phage-resistant *Campylobacter* strains from phage cocktail-administered chicks at a frequency of 13%. Strikingly, also before phages were applied resistance was observed, although at a lower frequency (6%). Nevertheless, an increase in the resistant *Campylobacter* population was observed after applying phages, suggesting that phages might have selected for resistant strains. Because further information on this topic is lacking, long-term efficacy of phages to control *C. jejuni* in poultry cannot be ensured.

### 1.3.5 Vaccination

Studies showed that if antibodies against *C. jejuni* are present in the chick, be it due to transfer of maternal antibodies to, or through immunization, the ability of *C. jejuni* to colonize these chicks is dramatically reduced (Sahin et al., 2003; Shoaf-Sweeney et al., 2008). This indicates that it may be possible to reduce *Campylobacter* colonization in broilers by immunization-based approaches (Shoaf-Sweeney et al., 2008). Several vaccination studies aiming to reduce the susceptibility of broiler chickens for *Campylobacter* colonization have been reported, although with variable results.

*In ovo* vaccination by injection of heat-killed *C. jejuni* in the amniotic fluid resulted in an increase in IgA antibodies (Noor et al., 1995). However, the consequences on a subsequent challenge were not studied. Intraperitoneal immunizations of chickens with killed *C. jejuni* whole cells at 16 and 29 days of age reduced the intestinal colonization, which was associated with an increase in specific IgY in intestinal secretions (Widders et al., 1996). In addition, Rice et al. (1997) demonstrated some reduction of *Campylobacter* colonization of chicks orally vaccinated with formalin-killed *C. jejuni* whole cells in combination with *Escherichia coli* heat-labile toxin when compared to non-vaccinated control birds.

For subunit vaccines, flagellin and OMPs have been tested and are considered useful candidates. In a study involving immunization of chickens with heat-killed *C. jejuni*, intestinal colonization upon challenge was reduced, with flagellin and a 67 kDa protein showing up as the immunodominant antigens (Widders et al., 1998). Vaccination of chickens with a hybrid protein, containing part of the *C. jejuni* FlaA and the B-subunit of *E. coli* heat-labile toxin, elicited specific antibodies against *C. jejuni* flagellin and reduced colonization of the chickens after challenge (Khoury & Meinersmann, 1995). Chickens orally immunized with an avirulent recombinant *Salmonella* strain carrying the *Campylobacter cjaA* gene, encoding a highly immunogenic lipoprotein which is conserved among different
Campylobacter serotypes, developed serum IgY and mucosal IgA antibody responses against Campylobacter and Salmonella OMPs and were protected against cecal colonization with a heterologous wildtype C. jejuni strain (Wyszynska et al., 2004). A more recent study evaluated the potential use of a heterologous vaccine for Campylobacter control in poultry using substantially more animals (Buckley et al., 2010). Upon vaccination with a Salmonella Typhimurium ΔaroA mutant, expressing CjaA as a plasmid-encoded fusion to tetanus toxin, birds had significantly reduced cecal C. jejuni counts of approximately log_{10} 1.4 cfu/g three and four weeks after C. jejuni inoculation, compared to unvaccinated control birds. This protection was associated with increased levels of CjaA-specific serum IgY and biliary IgA in the vaccinated chicks. Also in this study, a group of chicks receiving a vaccine strain containing the non-recombinant plasmid was incorporated. These animals were not protected, indicating that the protective effect observed in the birds receiving the heterologous vaccine, expressing CjaA, is due to responses directed against CjaA rather than competitive or cross-protective effects mediated by the carrier. Broiler chicks orally gavaged with live Salmonella-vectors expressing Campylobacter Omp18/CjaD, CjaA and ACE393 at day-of-hatch and inoculated with C. jejuni at 21 days of age, had higher serum IgG and mucosal sIgA levels, as well as reduced ileal C. jejuni counts at day 32 compared with control birds (Layton et al., 2010). Vaccination with the Omp18/CjaD peptide-expressed vector was most effective and Campylobacter could not be recovered from ileal samples. However, the cecal Campylobacter load, a better indicator for the colonization level in broilers (Beery et al., 1988), was not determined.

Zeng et al. (2009) showed that specific CfrA antibodies can block the function of this protein, diminishing ferric enterobactin-mediated growth promotion under iron-restricted conditions in a dose-dependent way. As inactivation of the cfrA gene completely eliminates Campylobacter colonization in chicks and CfrA is both expressed and immunogenic in chickens experimentally infected with C. jejuni, CfrA could be a promising candidate for a subunit vaccine for Campylobacter control in poultry (Zeng et al., 2009), but this hypothesis has yet to be tested.

Despite all this research, an effective vaccine to combat cecal Campylobacter colonization in poultry is not yet available. Recently, however, several critical colonization determinants have been identified which may be good targets for the development of effective vaccines to eradicate this zoonotic pathogen from poultry flocks. These potential vaccine candidates must be expressed during colonization in chicks and should ideally be highly immunogenic, conserved and prevalent among C. jejuni isolates. Bacterial OMPs are regarded as promising
vaccine components because of their accessibility for the host immune system and the key roles they play in the host-bacterium cross-talk (Zeng et al., 2009). CadF and CfrA may therefore hold much promise for such applications. Not only are they highly conserved and prevalent in C. jejuni strains, but these surface-exposed proteins are also highly immunogenic in chicks (Shoaf-Sweeney et al., 2008; Zeng et al., 2009). C. jejuni periplasmic PEB1 can possibly be transported across the outer-membrane and is highly immunogenic in humans. Whether this immunogenicity can be extended to the chicken host is not known. Finally, the secreted CiaB and transmembrane Tlp10 may be immunogenic but their precise role during chick colonization has yet to be determined. Also C. jejuni surface-exposed polysaccharide structures may be promising candidates for subunit vaccines. Indeed, several genes essential for successful colonization (kpsM, flaA, flgK, pglH, maf5 and cj1324) are involved in SACS biosynthesis. However, most SACS are highly variable and implicated in immune evasion in humans. Whether this can be extended to the chicken host is not clear. In any case, identification of conserved polysaccharide epitopes of SACS is critical for exploiting these structures for vaccine application. Finally, a plethora of other C. jejuni factors are indispensable for chicken gut colonization (see 1.2.2). Gene products of most of them are, however, not known to be surface-expressed, but rather reside in the peri- or cytoplasm where they exert their vital roles. As a consequence, they do not come in direct contact with the chick immune system and consequently are expected to have limited efficacy in vaccine applications.

1.3.6 Passive immunization

Experimental studies have shown that chick colonization can be inhibited by using antibodies. Campylobacter-specific maternal antibodies protect young chickens from colonization (Sahin et al., 2003). Pre-incubation of Campylobacter with rabbit hyper-immune antiserum or chicken bile antibodies increased the dose required to colonize the chicken cecum (Stern et al., 1990b). Oral administration of bovine or chicken Ig preparations from respectively milk or eggs of hyper-immunized animals, conferred a marked protection against challenge with C. jejuni in chickens (Tsubokura et al., 1997). Fecal bacterial counts were reduced by >99% (prophylaxis) or 80% - 95% (post-colonization) using an antibody preparation. The mean number of bacteria quickly increased, however, after ending the colonization-reducing addition with antibodies. This strategy might thus be applied to reduce cecal numbers of bacteria immediately before slaughter.
1.3.7 Competitive exclusion, probiotics and prebiotics

Although the exact exclusion mechanism is not fully understood, experiments have shown that competitive exclusion microflora can prevent *Campylobacter* colonization of the chicken gut. Competitive exclusion is a prophylactic measure that aims at increasing the resistance of chicks to *Campylobacter* infection.

Undefined bacterial mixtures have been demonstrated to effectively control *Campylobacter* infections in young chicks artificially challenged with a chicken *C. jejuni* isolate (Soerjadi *et al*., 1982; Soerjadi-Liem *et al*., 1984). In another study, however, this protective effect was not observed (Stern *et al*., 1988). The efficacy of competitive exclusion depends on cultivation methods and storage of the microbiota. It was found that the efficacy of using competitive exclusion microflora decreased with storage of the cultures (Stern, 1994). Different culture preparation techniques, with respect to the level of anaerobic culture, degree of epithelial scraping of the ceca, media used for subculturing and incubation temperature, resulted in different degrees of protection against colonization with *Campylobacter* spp. (Stern *et al*., 2001a). However, Schoeni & Wong (1994) concluded that protection by aerobically grown cultures was not statistically different from that obtained with anaerobically grown cultures.

Later, attempts have been made to develop defined microbiota. A standard feed supplemented with the yeast *Saccharomyces boulardii* did not significantly affect cecal *Campylobacter* colonization of experimentally challenged chickens (Line *et al*., 1998). The use of a probiotic containing *Lactobacillus acidophilus* and *Enterococcus faecium* in chicks, during the first three days of rearing, reduced both *C. jejuni* fecal shedding and jejunal colonization in colonized market-aged broilers, experimentally infected with *C. jejuni* six hours after the first oral administration of the probiotic, with 70% and 27% respectively (Morishita *et al*., 1997). Administration of competitive exclusion cultures of *Citrobacter diversus*, *Klebsiella pneumoniae* and *E. coli* effectively prevented or reduced *C. jejuni* colonization in chickens after *Campylobacter* inoculation (Schoeni & Wong, 1994). This protection was enhanced by feeding mannose to the chickens. In a simulated chicken digestive tract model, addition of *L. acidophilus*, *L. fermentum*, *L. crispatus* and *L. brevis* exerted an antagonistic effect on *C. jejuni* (Chang & Chen, 2000). A *Lactobacillus* strain was isolated from an adult chicken gut that showed bactericidal effects against *Campylobacter in vitro*, probably by the production of organic acids and an anti-*Campylobacter* peptide (Chaveerach *et al*., 2004a). Two promising antagonistic isolates (*L. salivarius* NRRL B-30514 and *Paenibacillus polymyxa* NRRL-B-
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30509), acting as probiotics, were ineffective to control Campylobacter, whether the isolates were fed to chicks before or after artificial challenge with C. jejuni (Stern et al., 2008). These isolates were, however, able to produce bacteriocins which are able to reduce the Campylobacter load in the gut of colonized birds (see further).

It has been demonstrated that it is possible to use combinations of (heterologous) C. jejuni chicken isolates for the competitive exclusion of human pathogenic C. jejuni strains in poultry (Chen & Stern, 2001). Circulation of uncharacterized environmental Campylobacter strains in commercial poultry flocks could possibly be biologically controlled by a characterized hyper-colonizing C. jejuni strain. Australian researchers identified such a strain that was capable of displacing other colonizing strains and maintain itself in the chicken GI tract for the entire 56-day broiler production cycle, without being displaced by other (hyper-)colonizing strains, once colonization was established (Calderon-Gomez et al., 2009).

With an approach called antibiotic dissection, day-old turkey poult’s were inoculated with cecal contents of Campylobacter-free adult turkeys after which the microbial communities in these poult’s were modified by different antibiotic treatments. It was investigated which modified intestinal microbiota was able to outcompete a Campylobacter challenge. Molecular examination of the constituents of these communities detected a subtype I of Megamonas hypermegale to be specific for a C. jejuni-suppressive application (Scupham et al., 2010). In vivo competition experiments with M. hypermegale isolates of both subtypes will be necessary to prove C. jejuni exclusion in poultry.

Finally, addition of mannanoligosaccharide to the feed of naturally infected birds and xylanase to the feed of artificially infected broilers, as prebiotics, resulted both in a minor, although significant decrease in cecal C. jejuni counts in these animals (Fernandez et al., 2000; Baurhoo et al., 2009).

1.3.8 Bacteriocin application

Svetoch & Stern (2010) recently reviewed bacteriocin application to reduce cecal Campylobacter counts in broiler chickens of colonized flocks. Applying purified encapsulated bacteriocin from either L. salivarius NRRL B-30514 or P. polymyxa NRRL-B-30509 to the feed during three days before euthanization led to a reduction of cecal Campylobacter colonization in broiler chickens, orally gavaged with C. jejuni at day-of-hatch, by at least six logs. However, birds were only seven to ten days of age and birds at slaughter age have not been examined in this study. Further research by these authors led to the identification of two
more bacteriocin-producing isolates with marked anti-
*Campylobacter* activity: *E. durans/faecium/hirae* (NRRL B-30745), producing bacteriocin BCN E 760 and *E. faecium* (NRRL B-30746), producing BCN E 50-52. Both bacteriocins were able to tremendously lower (> 6 log\(_{10}\) cfu/g or below detectable levels) the cecal *C. jejuni* load in inoculated broilers. Also in naturally-colonized market-aged broilers these bacteriocins were effective. BCN E 760 reduced the cecal *Campylobacter* load in these animals from an average of log\(_{10}\) 6.2 cfu/g to undetectable levels when added to the feed four days before slaughter. BCN E 50-52 at 10.8 mg per bird was able to reduce cecal colonization by > 5 log\(_{10}\) cfu/g when added to the drinking water three days before slaughter. Supplementing BCN 760 in drinking water at 3.5 to 25 mg per bird for three days before slaughter was most effective, resulting in a complete elimination of *C. jejuni* in 90% of the cases or else, a reduction of over six logs. The safety of these bacteriocins was confirmed by conducting experiments on monkey and human cell cultures as well as in treated mice and chickens. Santini *et al.* (2010) very recently reported both marked *in vitro* and *in vivo* activity for *Bifidobacterium longum* PCB 133 toward *Campylobacter*. After two weeks of daily administration, excreted *B. longum* PCB 133 counts were still high in the feces of orally gavaged chicks, even after a wash-out period of six days and *C. jejuni* numbers were significantly reduced by one log after this administration period.

### 1.4 GENERAL CONCLUSION

*Campylobacter jejuni* is the most common cause of bacterial-mediated diarrheal disease in humans worldwide and poses a serious health burden in industrialized countries. Despite their fragile nature, *Campylobacter* spp. are highly adapted to the broiler chicken gut. In contrast to the severe inflammatory disease that is often observed in humans, *C. jejuni* colonizes chickens asymptotically and colonized birds carry a very high bacterial load in their gastro-intestinal (GI) tract, especially the ceca. *C. jejuni* is typically detected in broiler flocks only starting from two to four weeks of age, which coincides with a reduction in maternally-derived anti-*C. jejuni* antibodies in these chicks. After this lag-phase, birds become more susceptible and colonization is detected in most flocks. Rapid transmission through the fecal-oral route results in colonization of all birds in a flock only a few days after the first bird is colonized. The inefficient immune response that is induced during commensal colonization with *C. jejuni* is not able to clear this zoonotic pathogen from the chicken gut. As a consequence, animals remain colonized up to slaughter which results in an enormous amount of contaminated
carcasses after processing. Increasing evidence suggests that *Campylobacter*-contaminated poultry meat is the most important source of campylobacteriosis in humans. While hygienic measures at the farm and control measures during carcass processing can have some effect on the reduction of *Campylobacter* numbers on the retail product, intervention at the farm-level by reducing colonization of the ceca should be definitely taken into account in the overall control policy. However, at present no effective strategy exists that consistently reduces *Campylobacter* counts in the ceca of broiler chicks. As a consequence, the prevalence of *Campylobacter* in broiler flocks and on broiler carcasses, and thus the incidence of campylobacteriosis in humans remain strikingly high.

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CHAPTER 2: AIMS
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Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease in humans worldwide and poses a serious health burden in industrialized countries. Poultry is considered the most important source for campylobacteriosis in humans. C. jejuni is highly adapted to the chicken gut and colonized birds carry a very high bacterial load, especially in their ceca. Slaughtering infected broiler flocks results in an enormous amount of contaminated carcasses after processing which can transmit the pathogen to humans. Since the cecum is the only amplification point of C. jejuni throughout the entire food chain, on-farm intervention measures that reduce colonization of the ceca may have a great impact on the number of campylobacteriosis cases in humans. The use of antimicrobial agents for this purpose is not allowed and at present no alternative strategy exists that consistently reduces C. jejuni in broiler flocks.

Therefore, the general scientific aim of this project was to develop an intervention measure that consistently reduces C. jejuni numbers in the ceca of broiler chickens by at least a thousand-fold, which would lead to a reduction in the number of human campylobacteriosis cases by a ten-fold (Rosenquist et al., 2003, Messens et al., 2007; Reich et al., 2008).

For this purpose,

- organic acids (chapter 3.1) and plant-derived antimicrobial compounds (chapter 3.2) were screened and tested for their anti-C. jejuni activity using several in vitro and in vivo models mimicking the C. jejuni-chicken host interaction;
- the potential contribution of passive immunization for C. jejuni control in broilers was investigated (chapter 3.3).
CHAPTER 3: EXPERIMENTAL STUDIES

3.1 EFFICACY OF WATER AND IN-FEED APPLICATIONS OF MEDIUM-CHAIN FATTY ACIDS ON CAMPYLOBACTER JEJUNI COLONIZATION AND TRANSMISSION IN BROILER CHICKENS

3.1.1 Intestinal mucus protects Campylobacter jejuni in the ceca of colonized broiler chickens against the bactericidal effects of medium-chain fatty acids

3.1.2 Application of medium-chain fatty acids in drinking water increases the Campylobacter jejuni colonization threshold in broiler chicks

3.2 THE CINNAMON-OIL INGREDIENT TRANS-CINNAMALDEHYDE FAILS TO TARGET CAMPYLOBACTER JEJUNI IN THE BROILER CHICKEN CECUM DESPITE MARKED ACTIVITY IN VITRO

3.3 PASSIVE IMMUNIZATION TO PREVENT AND REDUCE CAMPYLOBACTER JEJUNI COLONIZATION AND TRANSMISSION IN POULTRY
CHAPTER 3: EXPERIMENTAL STUDIES

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SUMMARY

Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease worldwide. As poultry and poultry products are a major source of C. jejuni infections in humans, efforts should be taken to develop strategies to decrease Campylobacter shedding during primary production. For this purpose, the efficacy of medium-chain fatty acids (MCFA) as feed additives to control C. jejuni colonization in broiler chickens was analyzed. First, the antimicrobial activity of the MCFA caproic, caprylic, and capric acid on C. jejuni was evaluated in vitro. Minimal inhibitory concentrations (MIC) were 0.25 mM for caproic and 0.5 mM for caprylic and capric acid at pH 6.0, and 4 mM for all 3 compounds at pH 7.5. Time-kill curves revealed strong bactericidal properties of the tested compounds toward C. jejuni at pH 6.0. Concentrations as low as 4 mM of caprylic and capric acid, and 16 mM of caproic acid killed all bacteria within 24 h. Capric acid had the highest activity, with concentrations of 4 mM killing all bacteria within the hour. Together these data show a profound bactericidal, dose-dependent activity of the tested MCFA toward C. jejuni in vitro. For this reason, the effect of these 3 MCFA on C. jejuni was evaluated in vivo. The addition of any of the acids to the feed, from 3 day before euthanization, was not capable of reducing cecal Campylobacter colonization in 27-day-old broilers, experimentally infected with C. jejuni at 15 days of age. Using a ‘cecal loop’ model, sodium caprate was not able to reduce cecal Campylobacter counts. When time-kill curves were conducted in the presence of chick intestinal mucus, capric acid was less active against C. jejuni. At 4 mM, all bacteria were killed only after 24 h. Thus, despite the marked bactericidal effect of MCFA in vitro, supplementing these acids to the feed does not reduce cecal Campylobacter colonization in broiler chickens under the applied test conditions, probably due to the protective effect of the mucus layer.

Key words: medium-chain fatty acid; Campylobacter jejuni; broiler chicken; chicken intestinal mucus
INTRODUCTION

Today, *Campylobacter jejuni* is the most common cause of bacterial-mediated diarrheal disease in humans, worldwide (Blaser, 1997; Zilbauer et al., 2008). Most *Campylobacter* infections are self-limiting, seldom requiring antimicrobial therapy. In a small number of cases however, an infection may lead to long-term complications of which Guillain-Barré syndrome, an acute autoimmune disease affecting the peripheral nervous system with an incidence of one in 1000 infected people, is the most serious (Butzler, 2004). Poultry and poultry products are considered one of the most significant sources for *Campylobacter* infections (Vellinga & Van Loock, 2002; Friedman et al., 2004; Wingstrand et al., 2006). It is suggested that chickens as a reservoir account for 50% to 80% of human campylobacteriosis cases (EFSA, 2010). Many efforts aimed at minimizing contamination of poultry with *Campylobacter* failed however to reduce the number of reported poultry meat consumption-related human campylobacteriosis cases (EFSA, 2009).

A significant correlation exists between *Campylobacter* concentrations in the intestinal contents and on chicken carcasses after processing (Herman et al., 2003; Rosenquist et al., 2003). Even small amounts of cecal contents (5 mg) can remarkably increase the numbers of *Campylobacter* on eviscerated broiler carcasses (Berrang et al., 2004) as *Campylobacter* are found abundantly in the large intestine, cloaca and ceca, where $10^5 - 10^9$ cfu/g fecal content have been observed (Corry & Atabay, 2001). A recent study stated that the number of campylobacteriosis cases in Belgium associated with consumption of chicken meals could be reduced by 84% by lowering *Campylobacter* numbers on chicken carcasses by 2 log (Messens et al., 2007), while a Danish quantitative risk assessment showed that this reduced contamination level would decrease the number of campylobacteriosis cases 30 times (i.e. a 97% reduction) (Rosenquist et al., 2003). This indicates that measures taken to reduce *Campylobacter* colonization in the intestinal tract of poultry during primary production have potential for reducing contamination of poultry products, and thus the incidence of human campylobacteriosis. For this purpose, a plethora of different approaches has been examined over the past few years, all with varying degrees of success. Amongst others, vaccination (Noor et al., 1995; Widders et al., 1996; Rice et al., 1997; Wyszynska et al., 2004) and passive immunization (Sahin et al., 2003) of chickens has been studied. Also, the effect of feeding broilers competitive exclusion microbiota (Soerjadi-Liem et al., 1984; Schoeni & Wong, 1994; Stern et al., 2001), bacteriophages (Wagenaar et al., 2005), bacteriocins (Line et al., 2008), organic acids (Byrd et al., 2001; Chaveerach et al., 2004; Van Deun et al., 2008a).
or their derivatives (Hilmarsson et al., 2006), and antibiotics (Farnell et al., 2005) have been assessed. Nonetheless, to date there is no effective, reliable, and practical intervention measure available to reduce colonization of the broiler gut with Campylobacter (Lin, 2009). Antibacterial growth promoting agents used in the rearing of broiler chickens have been banned by the European Union due to concerns regarding the use of chemical additives in food production (Dibner & Richards, 2005). Also for therapeutic purposes, the use of antibacterial agents should not be encouraged. Indeed, there is increasing evidence of the development of antimicrobial resistant Campylobacter strains which may compromise the treatment of human campylobacteriosis (Smith et al., 1999; Engberg et al., 2001; Rao et al., 2005; Gibreel & Taylor, 2006; Moore et al., 2006; Luangtongkum et al., 2009). Using fatty acids instead could be a promising alternative because of their antimicrobial activities against a wide range of microorganisms (Kabara et al., 1972; Bergsson et al., 2002; Van Immerseel et al., 2004a; Boyen et al., 2008), including Campylobacter (Thormar et al., 2006; Houf et al., 2007). Van Immerseel et al. (2006) stated that of all fatty acids, medium-chain fatty acids (MCFA) have probably the greatest antimicrobial activity against Salmonella, which like Campylobacter is a Gram-negative bacterium causing gastro-enteritis. Recently, it was shown that the supplementation of the MCFA caprylic acid to broiler feed could reduce C. jejuni colonization in these animals, both in a prophylactic way (Solis de los Santos et al., 2008a) as well as therapeutically (Solis de los Santos et al., 2008b; Solis de los Santos et al., 2009).

The aim of this study was to investigate the applicability of MCFA to obtain a 3 log reduction in cecal Campylobacter levels in infected chickens before slaughter, thereby aiming at lowering carcass contamination and eventually the number of human campylobacteriosis cases. First, a thorough in vitro evaluation of the antimicrobial activity of MCFA on C. jejuni was performed. Secondly, the in vivo effect of these acids toward C. jejuni was analyzed.

MATERIALS AND METHODS

Experimental animals

Day-of-hatch Ross broiler chickens of both sexes from a local farm were raised in group until treatment and provided with a commercial starter feed and water ad libitum. Husbandry, euthanasia methods, experimental procedures and bio-safety precautions were approved by the Ethical Committee (EC) of the Faculty of Veterinary Medicine, Ghent University, Ghent,
Belgium (EC numbers: 2009/31, 2009/56 and 2009/114wi). Chicks were examined for the presence of *Campylobacter* in mixed fecal samples and proved to be *Campylobacter* free.

**Bacterial strains and culture conditions**

*Campylobacter jejuni* strains KC 40 and KC 96.1 from poultry origin as well as R-27451, R-27456, R-27467 and R-27473 (kindly provided by Prof. Dr. Peter Vandamme, Ghent University, Ghent, Belgium) isolated from human patients suffering from campylobacteriosis, were used to perform MIC assays. These strains were chosen based on their difference in invasion efficiency in primary cecal epithelial cells from chickens, as determined by Van Deun et al. (2008b). For all further experiments, *C. jejuni* strain KC 40 was used. This strain colonizes chickens to a high level (Van Deun et al., 2008b). Bacteria were routinely cultured in Nutrient Broth No.2 (NB2, CM0067; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with Modified Preston *Campylobacter*-selective supplement (SR0204E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), at 42°C under microaerobic conditions (5% O₂, 5% CO₂, 5% H₂, 85% N₂). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank’s Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on modified charcoal cefoperazone deoxycholate agar (mCCDA, CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), followed by microaerobic incubation at 42°C for 22 h.

**Collection of chicken intestinal mucus**

Commercial 28-week old brown laying hens were euthanized and the small intestine was collected and gently rinsed with PBS to remove fecal material. The mucus was scraped from the mucosa with a glass slide covered in parafilm, diluted 1:3 with N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES, 25 mM, pH 7.4) and vortexed. The solution was centrifuged three times at 2000 × g for 15 min at 4°C. The supernatant containing the crude mucus was centrifuged two times more at 12,000 rpm for 15 min at 4°C and filter sterilized by passage through a 0.45 µm pore size filter (IWAKI, International Medical, Brussels, Belgium) and stored at -80°C. Protein content was determined using a Biorad protein assay kit (Biorad, Nazareth, Belgium).
**Minimal inhibitory concentrations of medium-chain fatty acids against C. jejuni**

MIC values of MCFA against *C. jejuni* were determined using a microdilution method following the Clinical and Laboratory Standards Institute guideline M31-A3 (CLSI, 2008) with some minor modifications. MIC values of MCFA were determined in triplicate at pH 6.0 and pH 7.5 using HCl or NaOH to obtain the desired pH-values. Caproic (C\(_6\):0), caprylic (C\(_8\):0) and capric (C\(_{10}\):0) acid (all products from Sigma, St. Louis, MO) were tested after serial 2-fold dilutions in 96 microtiter well plates in Mueller-Hinton (MH) broth (CM0405; Oxoid), without the addition of lysed horse blood, ranging from 0.063 to 16 mM. These tested concentrations were chosen based on preliminary studies (data not shown) conducted in our laboratory. Where needed, acids were fully dissolved into the broth by heating (56°C). Inoculum was prepared by collecting colonies directly from blood agar plates that were incubated for 24 to 48 h at 42°C in a microaerobic environment, suspending them into sterile PBS until a turbidity equivalent to a 0.5 McFarland standard was achieved, and diluting 1:4 in MH broth. Five µL of this suspension was inoculated into 195 µL medium in each well, so the final inoculum contained approximately 10\(^5\) cfu/mL. These suspensions were incubated for 24 h at 42°C in a microaerobic environment (as above) after which microbial growth was assessed. Where needed, plates were incubated for an additional time until growth was observed for the positive controls. The lowest concentration completely inhibiting visible growth was considered the MIC.

**Time-kill curve experiments**

Time-kill curve experiments were conducted in MH broth supplemented with the MCFA caproic, caprylic, or capric acid at 1, 4, and 16 mM or without acid (growth control). The solutions were brought to pH 6.0 using HCl or NaOH followed by inoculation with a *C. jejuni* suspension grown for 17 h in NB2 with supplements, so the *C. jejuni* concentration in these solutions was approximately 10\(^7\) cfu/mL. Bacterial counts were determined at 0, 1, 2, 4, 8, 16, and 24 h post-inoculation (pi). The experiment was conducted in triplicate.

Time-kill curve experiments in HBSS supplemented with 5 mg/mL chicken intestinal mucus and capric acid at 1, 4, and 16 mM or without capric acid (growth control), were conducted analogously.
Supplementation of non-coated medium-chain fatty acids to broilers experimentally inoculated with *C. jejuni*

Day-of-hatch broiler chicks (n = 50) were raised in group. At the age of 15 d, all chicks were orally inoculated with approximately $5 \times 10^7$ cfu of tylosin-sensitive *C. jejuni* strain KC 40. At 23 days of age, the chicks were randomly assigned to 5 treatment groups (n = 10 per group): negative control (*Campylobacter* infected, no MCFA); positive treatment control (*Campylobacter* infected, 1% (wt/wt) tylosin granules (TYLAN 100 Granules, Elanco, Brussels, Belgium)); and 3 acid treatment groups provided with 1% (wt/wt) non-coated caproic, caprylic, or capric acid sodium salt (all products from Sigma) in the feed. From this moment, chickens were housed in groups in separate isolating chambers. Acids and tylosin granules were added for the last 72 h of the 28-day trial. Equal amounts of feed were provided for each group during treatment and care was taken that all animals had unlimited access to the feed. After treatment the chicks were euthanized by injection of T61 IV in the wing vain (600 µL/chick), and the ceca as well as their contents were collected for *C. jejuni* enumeration (see below).

Supplementation of coated medium-chain fatty acids to broilers experimentally inoculated with *C. jejuni*

An *in vivo* trial with coated MCFA was performed analogously to the experiment with non-coated MCFA. The chicks were randomly assigned to 4 treatment groups (n = 10 per group): negative control (*Campylobacter* infected, no MCFA); tylosin-control (*Campylobacter* infected, 1% tylosin granules); and 2 acid treatment groups provided with coated caproic or caprylic acid (all products from Sanluc International nv, Oosterzele, Belgium.), at final concentrations of the pure acids in the feed of 1% (wt/wt). Coated capric acid could not be obtained for this study.

Cecal loop model

A ‘cecal loop’ model was used to examine the effect of the sodium salt of caprylic acid on cecal *Campylobacter* numbers. This model allows direct comparison of *C. jejuni* numbers in both ceca of the same animal. One cecum (positive control) is injected with a control solution, while in the other cecum a test compound is injected (Van Deun et al., 2008b). Day-of-hatch broiler chicks (n = 6) were raised in group. At the age of 14 days, chicks were divided in two groups (n = 3) and housed in separate isolation units. At the age of 15 days, all chicks of group 1 were inoculated with approximately $3.5 \times 10^7$ cfu/mL of *C. jejuni* strain KC 40, while
the chicks of group 2 were kept in a *Campylobacter* free environment. At the age of 21 days, cecal loops were applied in all six animals. Twelve h before surgery, buprenorfine was administered. Chicks were anaesthetized with isoflurane and the abdominal cavity was opened with an incision of two inch, caudal of the sternum. The ceca were exposed and one loop in each cecum was constructed using Vicryl 4/0 surgical suture. One of the ceca of each animal of group 1 was injected with 200 µL 75 mM sodium caprate (Sigma) in HBSS, while the other cecum was injected with 200 µL HBSS, and thus served as control. In the animals of group 2, one cecum was injected with 100 µL 40 mM sodium caprate in HBSS, while the other cecum was injected with 100 µL HBSS. After this, both ceca were injected with 100 µL of a *C. jejuni* KC 40 suspension of approximately $2 \times 10^5$ cfu/mL in HBSS. After injection, the cecum was repositioned in the abdominal cavity and the peritoneum, muscles and skin were sutured. After 24 h the chickens were euthanized by intravenous injection with T61 and the ceca with their contents were collected for cecal *Campylobacter* enumeration (see below).

**Cecal Campylobacter enumeration**

Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with supplements. After homogenization, a 10-fold dilution series was made in HBSS. Of each dilution, 100 µL was spread on mCCDA plates. After 22 h incubation at 42°C under microaerobic conditions, colonies were counted. For enrichment, diluted cecal samples in NB2 were incubated at 37°C under microaerobic conditions. After 24 h, samples were plated on mCCDA and incubated at 42°C in a microaerobic environment. After 24 and 48 h plates were examined for the presence or absence of *C. jejuni*.

**Statistical analysis**

Data of the *in vivo* trials and the ‘cecal loop’ experiment were analyzed by SPSS 17.0 software for Windows. The significance level $\alpha$ was set at 0.05. *Campylobacter* counts were first transformed to $\log_{10}$ counts before statistical analysis. A one-way analysis of variance (ANOVA) was then carried out to compare the means of $\log_{10}$ transformed counts in chicken cecal contents of all groups (treated groups and control group) in the *in vivo* trials. Significant differences were assessed by Bonferroni Post Hoc tests. $P$-values below 0.05 were considered significantly different. A paired-samples $t$-test was performed to compare the means of $\log_{10}$ transformed counts in chicken cecal contents of the treated group and control group in the ‘cecal loop’ experiment. $P$-values below 0.05 were considered significantly different.
RESULTS

Minimal inhibitory concentrations of medium-chain fatty acids against \textit{C. jejuni}

Table 1.1.1 shows the results of the determination of the MIC values of the MCFA caproic, caprylic, and capric acid for two poultry and four human derived \textit{C. jejuni} strains.

Table 1.1.1 Overview of the minimal inhibitory concentration (MIC) of caproic, caprylic and capric acid, tested in Mueller-Hinton (MH) broth at pH 6.0 and 7.5 against 2 chicken and 4 human derived \textit{Campylobacter jejuni} strains

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<th>Compound</th>
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MIC value variation for each strain for a given acid was maximally one 2-fold dilution. The MIC of all fatty acids was 4- to 16-fold lower at pH 6.0 compared to pH 7.5 and was comparable for all three MCFA.

Time-kill curves of medium-chain fatty acids against \textit{C. jejuni}

Based on the MIC values obtained in the previous experiment, time-kill curves were prepared to analyze the bactericidal effect of MCFA toward \textit{C. jejuni} strain KC 40 using 1, 4 and 16 mM at pH 6.0, over a 24-h period. MH broth without acids and adjusted to pH 6.0 served as a growth control.

Colony count variation at each time point for each concentration, between triplicate experiments, was less than 10%. All three MCFA at 1 mM were only moderately bactericidal (< 2 logs kill at any time point) toward \textit{C. jejuni} (figure 1.1.1). After 24 h, bacterial counts approached or exceeded the initial inocula but were lower than the growth control counts for
Figure 1.1.1 Time-kill curves of medium-chain fatty acids (MCFA) at pH 6.0 against Campylobacter jejuni strain KC 40. (A) caproic acid, (B) caprylic acid, and (C) capric acid. Bacterial counts are represented as log₁₀ cfu/mL. ♦, growth control; □, MCFA at 1 mM; ▲, MCFA at 4 mM; ○, MCFA at 16 mM. The experiment was repeated 3 times, and the average numbers are shown.

all acids. The same result is observed for caproic acid at 4 mM (< 1 log kill at any time point). At 4 mM, caprylic acid killed all C. jejuni bacteria within 16 h after exposure, whereas capric acid killed all bacteria within 1 h pi at this concentration. At 16 mM, all three MCFA reduced
bacterial counts below the detection limit (within 16 h pi for caproic acid and within 1 h pi for caprylic and capric acid) and maintained these numbers after 24 h. At 4 and 16 mM, capric acid was bactericidal already within seconds, as proven by strongly reduced bacterial numbers shortly (at 0 h) after exposure.

**Feed supplemented with coated or non-coated medium-chain fatty acids does not reduce cecal *C. jejuni* colonization in broilers**

Feed supplemented with non-coated (i.e. free) sodium caproate, caprylate or caprate did not significantly ($P > 0.05$) reduce cecal *Campylobacter* load in chickens, compared with the feed without supplements (*figure 1.1.2*). Cecal *Campylobacter* counts of animals fed tylosin-supplemented feed was again significantly ($P < 0.05$) reduced by approximately 3.5 logs, compared to the control group (3.9 ± 0.7 and 7.4 ± 0.6 log$_{10}$ cfu/g, respectively). Feed uptake (data not shown) was similar for all groups during the treatment period.

![Figure 1.1.2](image)

*Figure 1.1.2* Cecal *Campylobacter jejuni* counts in 27-day-old broilers (n = 10 chickens/treatment) fed un-supplemented feed, tylosin, and sodium caproate, caprylate, or caprate from 3 days before euthanization. Values are represented as log$_{10}$ cfu/g cecal contents and are mean ± standard deviation (n = 10). An asterisk denotes significantly lower cecal *Campylobacter* levels compared to the control group ($P < 0.05$).

Feed supplemented with coated caproic or caprylic acid did not significantly ($P > 0.05$) alter cecal *Campylobacter* counts in broilers, compared with the un-supplemented feed (*figure 1.1.3*). Cecal *Campylobacter* load of chickens fed tylosin-supplemented feed was significantly
Experimental studies

\( (P < 0.05) \) reduced by approximately 4 logs, compared to the control group (2.8 ± 1.5 and 7.0 ± 0.8 \( \log_{10} \) cfu/g, respectively). Feed uptake (data not shown) was similar for all groups during the treatment period.

![Graph showing cecal Campylobacter jejuni counts](image)

**Figure 1.1.3** Cecal *Campylobacter jejuni* counts in 27-day-old broilers (n = 10 chickens/treatment) fed un-supplemented feed, tylosin, and coated caproic or caprylic acid from 3 days before euthanization. Values are represented as \( \log_{10} \) cfu/g cecal contents and are mean ± standard deviation (n = 10). An asterisk denotes significantly lower cecal *Campylobacter* levels compared to the control group (\( P < 0.05 \)).

**Sodium caprate is not able to reduce cecal C. jejuni numbers in the ‘cecal loop’ model**

The cecal content of 21-day-old broilers was estimated to be 1.0–2.0 mL. By injecting 100 µL 40 mM (as well as 100 µL *Campylobacter* suspension) or 200 µL 75 mM sodium caprate, final caprate concentrations in these ceca ranged then from 1.8–3.3 mM or 6.8–13 mM, largely sufficient to cause a growth-inhibitory, respectively bactericidal effect toward *C. jejuni* under *in vitro* conditions (see above). Despite these concentrations being present, sodium caprate did not significantly (\( P > 0.05 \)) alter the mean cecal *Campylobacter* levels in the cecal loops of colonized (B) or non-colonized (A) broilers compared with the control cecum after 24 h (figure 1.1.4). There was some variability in the results however, with one bird in each study showing an approximate 2 log reduction in cecal *C. jejuni* counts, while in the other two birds there was a slight increase (or decrease), so that overall no significant effect of the treatments could be observed.
Experimental studies

Figure 1.1.4 *Campylobacter jejuni* counts in cecal loops of 22-day-old broilers (n = 3 chickens/treatment). In (A), one cecum of a *Campylobacter*-free chick was injected with 100 µL 40 mM sodium caprate (L), the other with 100 µL HBSS (R). Both ceca were injected with 100 µL of a 2 × 10^5 cfu/mL *C. jejuni* suspension and *C. jejuni* counts were determined 24 h later. In (B), one cecum of a *C. jejuni*-infected chick was injected with 200 µL 75 mM sodium caprate (L), the other with 200 µL HBSS (R) and *C. jejuni* counts were determined 24 h later. Values are represented as log_{10} cfu/g cecal contents. Also the mean ± standard deviation (n = 3) is given. Each symbol represents *C. jejuni* counts in both ceca of the same bird.

**Chicken intestinal mucus protects *C. jejuni* against anti-*Campylobacter* activity of capric acid**

Figure 1.1.5 shows the influence of chicken intestinal mucus on growth and survival of *C. jejuni* KC 40 in the presence of capric acid at pH 6.0. Bacterial counts were determined at different time intervals in MH broth (A) or in HBSS with 5 mg/mL chicken intestinal mucus (B). MH broth, respectively HBSS with 5 mg/mL mucus at pH 6.0 without capric acid served as the growth controls.
Figure 1.1.5 Influence of chicken intestinal mucus on growth and survival of Campylobacter jejuni KC 40 in the presence of capric acid at pH 6.0. Time kill curves are shown for capric acid in (A) MH broth, and (B) HBSS supplemented with 5 mg/mL chicken intestinal mucus. Bacterial counts are represented as log$_{10}$ cfu/mL. ♦, growth control; □, capric acid at 1 mM; ▲, capric acid at 4 mM; ○, capric acid at 16 mM. The experiment was repeated 3 times, and the means are shown.

Colony count variation at each time point for each concentration, between triplicate experiments, was less than 10%. Mucus had no effect on the bactericidal properties of capric acid at 16 mM. Bacterial counts were reduced under detectable levels within the hour whether (B) or not (A), mucus was added. Also capric acid at 4 mM killed all bacteria within the hour without mucus (A), while this reduction was only obtained after 24 h in the presence of mucus (B). At 1 mM capric acid was growth-inhibitory toward C. jejuni in the absence of mucus (A), but not when mucus was added (B).
DISCUSSION

The first objective of this study was to evaluate the antimicrobial activity of MCFA against *Campylobacter jejuni in vitro*. MIC values of MCFA toward *C. jejuni* were pH-dependent, probably attributable to the increased dissociation of the acids in neutral environments (Davidson, 1997). This was already reported to be the case for *C. jejuni* (Skrivanova et al., 2007) as well as for other bacteria (Sun et al., 1998; Boyen et al., 2008). Although the mode of action of MCFA and fatty acids in general on *C. jejuni* is still uncertain, the acids probably act by diffusing into bacterial cells in the un-dissociated form where they later on dissociate in the near-neutral environment of the bacterial cytoplasm, as hypothesized by Sun *et al.* (1998) for butyric acid. These accumulating anions lead to intracellular acidification, which can eventually cause killing of the bacterium (Russell, 1992; Sun et al., 1998). The observed MIC values of MCFA were at least 10-fold lower compared to the MIC of the short-chain fatty acid (SCFA) butyrate (6.25 mM in Brain Hart Infusion broth) as determined by Van Deun *et al.* (2008a) against *C. jejuni* KC 40 at pH 6.0. Lower MIC values for MCFA compared to SCFA can also be observed for *Salmonella* Typhimurium (Boyen et al., 2008), as well as for several other Gram-positive and -negative bacteria (Nakai & Siebert, 2003). Time-kill curve experiments revealed pronounced bactericidal properties of MCFA toward *C. jejuni* at pH 6.0, which approaches the mean cecal pH of broilers during their life-cycle (van der Wielen et al., 2000), with concentrations of 4 mM being able to reduce bacterial numbers under detectable levels after 16 h for caprylic and capric acid. The same result was achieved for caproic acid at 16 mM. At this concentration, caprylic and capric acid are strongly bactericidal within minutes. This is in accordance with Thormar *et al.* (2006), who showed that a 10-min incubation period in 10 mM caprylic and capric acid in aerobic broth at 37°C decreased *C. jejuni* counts by approximately $\log_{10} 3.5$ and $\log_{10} 7$, respectively (pH unreported). Also Skrivanova *et al.* (2007) found similar results: a 10-min incubation period in 1 mg/mL capric acid ($\approx 5.8$ mM) reduced *C. jejuni* levels from $> 10^9$ cfu/mL below the detection limit, both at pH 4.5 and 7.0. Hence, our results show a profound bactericidal, dose-dependent activity of the tested MCFA toward *C. jejuni in vitro* which may indicate a promising role for these compounds as possible agents to reduce cecal *C. jejuni* counts in colonized broiler chickens.

Therefore, our second objective was to test the antimicrobial activity of MCFA against *C. jejuni in vivo*. First, an experiment using free and micro-encapsulated MCFA was performed. Coating MCFA on micro-beads prevents absorption of the acids in the upper tract and results in a slow release of the acids during transport along the gastro-intestinal tract, allowing them
to reach the small intestine and the ceca (Van Immerseel et al., 2004b). This is of great importance because the latter represents the predominant site for Campylobacter colonization, where the bacterium is found deep in the cecal crypts within the mucus layer (Beery et al., 1988). For this reason, only the ceca were considered for analysis. MCFA concentrations of 1% were used in this trial, as Solis de los Santos et al. (2008b) found consistent results when using 0.7% or 1.4% caprylic acid. Coated capric acid could not be obtained for this experiment. Coated MCFA-supplemented feed, as well as non-coated MCFA sodium salts did not reduce cecal Campylobacter counts in experimentally infected chicks when administered three days before euthanization. Sodium salts (solid at room temperature) of MCFA were used for practical convenience and are believed to react in the same way as their acids (liquid at room temperature, except capric acid), because in vitro experiments conducted at our laboratory showed similar bactericidal properties against C. jejuni for both formulations (data not shown). Tylosin, a macrolide antibiotic that has been banned for use as a growth promoter by the EU Commission in 1999 (Dibner & Richards, 2005), and used as a positive treatment control, reduced cecal Campylobacter levels markedly in both in vivo experiments, pointing towards the successful performance of these tests.

For caprylic acid, our results are not in agreement with those obtained by Solis de los Santos et al. (2008b, 2009). These authors demonstrated a decrease in cecal C. jejuni counts of approximately 3 to 4 logs when supplementing feed with 0.7% caprylic acid 3 days before necropsy, both in 15-day-old (Solis de los Santos et al., 2008b) and 42-d-old (market-aged) (Solis de los Santos et al., 2009) experimentally infected broilers. Possibly, these contradictory observations could be attributed to 1) a difference in formulation of the acid in the feed, allowing it to reach the ceca at higher concentrations in the experiments of Solis de los Santos and coworkers; or 2) C. jejuni strain-specific properties, as we inoculated the chicks with a highly invasive strain that might be less susceptible toward the effects of MCFA in the broiler cecum compared to less invasive strains; or 3) other factors, such as differences in genetic background of the birds used in the 2 studies or differing dietary influences on cecal biochemistry. Next to these contradictory results, also the addition of 0.12% or 0.24% emulsified monocaprin, the 1-monoglyceride of capric acid, to drinking water and feeds of colonized birds was shown to significantly reduce fecal Campylobacter counts in these animals with 1 to 2 logs after 2-3 days (Hilmarsson et al., 2006). Since monocaprin is probably hydrolyzed into capric acid and glycerol by lipases in the digestive tract (Hilmarsson et al., 2006), this reduction is most likely attributed to the bactericidal properties of the former.
To determine whether capric acid, having the highest *Campylobacter* reduction activity *in vitro*, is capable of reducing *Campylobacter* numbers in the ceca of broiler chickens at concentrations that are bactericidal *in vitro*, the sodium salt of capric acid was tested in a cecal loop model. Sodium caprate was used because of its better solubility in aqueous solutions over capric acid, so higher concentrations could be injected in the ceca. Sodium caprate failed however to inhibit the growth of, or to kill *Campylobacter* in the broiler chicken cecum at concentrations that are growth-inhibitory or bactericidal *in vitro*, respectively.

Together these results suggest that *C. jejuni* probably is occupying a protected niche in the ceca of broilers, rendering them less susceptible to the bactericidal effects of capric acid seen *in vitro*. The mucus layer, which covers the entire surface of the chicken intestinal tract and is in fact seen as a part of the innate host response (Forstner *et al*., 1995), definitely plays a role herein since capric acid was less effective in inhibiting the growth of, and killing *C. jejuni* in the presence of chicken intestinal mucus. This was also already observed for the sodium salt of the SCFA butyric acid (Van Deun *et al*., 2008a). Whether this decreased susceptibility is caused by interference between mucosal constituents and the antimicrobial potency of these acids, or an indirect effect on the *C. jejuni* bacteria is not clear. Thus, where the chicken intestinal mucus layer usually is regarded as an anti-bacterial barrier by protecting against luminal microflora (Forstner *et al*., 1995), it seems that in the case of *Campylobacter* it forms a protective and supportive environment making it hard to eradicate this bacterium from the chicken intestinal tract, once colonized.

In conclusion, our results show strong bactericidal properties of MCFA toward *C. jejuni* *in vitro*, as previously reported. However, under the test conditions applied, MCFA were not effective at decreasing cecal *C. jejuni* counts in experimentally infected broilers and in the cecal loop model due to the protective effect of intestinal mucus. Most likely the concentrations applied were too low to target *C. jejuni* in its protective environment. Another strategy is to prevent *C. jejuni* colonization in broilers by administering MCFA preventively, from day-of-hatch. Indeed, van Gerwe *et al*. (2009) showed recently that the addition of a MCFA mixture to the feed at 1% reduces the probability of broilers to become colonized with *C. jejuni*.

To conclude, the application of MCFA cannot be advocated unambiguously to control *C. jejuni* in broilers until further studies have resolved apparently contradictory results.

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Experimental studies


CHAPTER 3: EXPERIMENTAL STUDIES

3.1. EFFICACY OF WATER AND IN-FEED APPLICATIONS OF MEDIUM-CHAIN FATTY ACIDS ON \textit{CAMPYLOBACTER JEJUNI} COLONIZATION AND TRANSMISSION IN BROILER CHICKENS

3.1.2. Application of medium-chain fatty acids in drinking water increases the \textit{Campylobacter jejuni} colonization threshold in broiler chicks

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SUMMARY

Campylobacteriosis is the most reported bacterial-mediated gastro-enteritic disease in many developed countries. Broiler chickens are a natural host for *Campylobacter* spp. and contaminated poultry meat products are a major source for transmitting pathogenic *Campylobacter* strains to humans. Currently, no intervention measure efficiently and effectively controls this pathogen in poultry flocks. Medium-chain fatty acids (caproic, caprylic, capric and lauric acid) show a marked anti-*Campylobacter* activity *in vitro*. However, in recent trials using our *in vivo* models, administering these acids to the feed of broiler chicks did neither prevent, nor reduce cecal *C. jejuni* colonization in broilers. In the present study we examined whether a drinking water application of medium-chain fatty acids might be more effective in combating *Campylobacter* colonization in poultry. Although *Campylobacter* colonization and transmission was not reduced, we demonstrate that adding an emulsion of a mixture of caproic, caprylic, capric and lauric acid to the drinking water of broiler chicks reduces their colonization susceptibility and prevents *C. jejuni* survival in drinking water. Thus, the merit of water applications of medium-chain fatty acids is the reduction of the probability of *Campylobacter* entry into and transmission throughout a flock.

**Keywords:**
*Campylobacter jejuni*; broiler chicken; medium-chain fatty acid; water application; colonization threshold
INTRODUCTION

Campylobacteriosis is the most reported zoonotic disease in many developed countries, with in particular *Campylobacter jejuni* as the causative agent (EFSA, 2010a). Broiler chickens are a natural host for this pathogen and their carcasses often become contaminated during slaughter. Poultry products are therefore considered one of the most significant sources for campylobacteriosis in humans (Friedman *et al.*, 2004; Vellinga & Van Loock, 2002; EFSA, 2010b). Hygiene and biosecurity efforts aimed at minimizing contamination of poultry with *Campylobacter* seem not to be effective. Furthermore, no reliable and practical intervention measure is currently available to reduce *Campylobacter* colonization of the broiler gut (Hermans *et al.*, 2011a).

For this purpose, the use of medium-chain fatty acids (MCFA) might be promising due to their marked antibacterial activity against *C. jejuni in vitro*, which was especially observed for capric acid. Furthermore, the antibacterial potency of MCFA is believed to exceed that of short-chain fatty acids (SCFA) (Hermans *et al.*, 2010). Several recent studies described the evaluation of in-feed SCFA (formic, acetic, propionic and butyric acid) and MCFA (caproic, caprylic, capric and lauric acid) to reduce (Hermans *et al.*, 2010; Molatova *et al.*, 2010; Solis de los Santos *et al.*, 2010) or prevent (Van Deun *et al.*, 2008; Solis de los Santos *et al.*, 2008) cecal *Campylobacter* colonization in broilers; however, inconsistent results have been reported. Alternatively to their in-feed use, MCFA can be administered through the drinking water as well, but studies using water applications of MCFA are scarce. Although only poorly soluble in aqueous solutions, these acids can be mixed in the drinking water by creating MCFA emulsions (Hilmarsson *et al.*, 2006). These emulsions may exert their bactericidal properties on *C. jejuni* in the drinking water, at the crop and in the stomach of the animals, in this way providing a barrier for bacterial entry in the chicken gut. Therefore, the aim of this study was to investigate the effect of a (1:1) commercial MCFA/water emulsion in the drinking water (0.4% vol/vol) of broiler chickens on *Campylobacter* susceptibility of birds, transmission and cecal survival.

MATERIALS AND METHODS

Experimental animals

Day-of-hatch Ross broiler chickens of both sexes from a local farm were raised individually and provided with a commercial starter feed *ad libitum*. Husbandry, euthanasia methods,
experimental procedures and bio-safety precautions were approved by the Ethical Committee (EC numbers 2009/114wi and EC2010/110) of the Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium. Chicks were examined for the presence of Campylobacter in mixed fecal samples prior to inoculation and proved to be Campylobacter free.

**Bacterial strains and culture conditions**

*Campylobacter jejuni* strain KC 40 from poultry origin was used for all experiments. This strain colonizes chickens to a high level (Van Deun *et al.*, 2008; Hermans *et al.*, 2010). Bacteria were routinely cultured in Nutrient Broth No.2 (NB2, CM0067; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with Modified Preston Campylobacter-selective supplement (SR0204E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), at 42°C under microaerobic conditions (5% O₂, 5% CO₂, 5% H₂, 85% N₂). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank’s Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on modified charcoal cefoperazone deoxycholate agar (mCCDA, CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), followed by microaerobic incubation at 42°C for 22 h.

**Anti-*C. jejuni* effect of the MCFA emulsion in vitro**

The effect of a commercial MCFA emulsion (delivered by Vitamex, Drongen, Belgium), containing approximately 50% MCFA (a mixture of caproic, caprylic, capric and lauric acid), on *C. jejuni* strain KC 40 survival was assessed (Deschepper *et al.*, 2003). The MCFA emulsion was diluted in tap water to a final concentration of 0.4% (vol/vol), the dose recommended by the manufacturer. Tap water without MCFA was used for control. Water samples were inoculated with a *C. jejuni* suspension grown for 17 h, so the final starting inoculum was approximately 10⁷ cfu/mL, and incubated at 25°C under aerobic conditions. After 1 minute of incubation *C. jejuni* counts were enumerated.

**Effect of the MCFA emulsion on established *C. jejuni* colonization in four-week-old broiler chicks**

Twenty-four chicks were raised in group and received a commercial starting feed and drinking water *ad libitum*. The animals remained on the same feeding and drinking regime for the entire duration of the experiment except for the last three days. After two weeks all animals were orally challenged with approximately 10⁶ cfu *C. jejuni* KC 40. At 25 days of
age, birds were assigned to two treatment groups (n = 12 birds/group). Animals of group 1 received standard drinking water, while those of group 2 received water supplemented with 0.4% (vol/vol) MCFA emulsion until the end of the experiment (trial 1). After four weeks all birds were euthanized by injection of T61 IV (Intervet, Belgium) in the wing vein, and the ceca as well as their contents were collected for *Campylobacter* enumeration (see below).

**Effect of the MCFA emulsion on *Campylobacter* colonization susceptibility of individually housed broiler chicks**

Day-of-hatch broiler chicks (n = 60) were housed individually and randomly assigned to six groups. Animals of groups 1 to 3 received standard drinking water, while those of groups 4 to 6 received water supplemented with 0.4% (vol/vol) MCFA emulsion. All animals received a commercial starting feed *ad libitum* and animals remained on the same feeding and drinking regime for the entire duration of the experiment. At 14 days of age, all birds were orally inoculated with 1 mL of $3 \times 10^2$ (groups 1 and 4), $2 \times 10^3$ (groups 2 and 5) or $2 \times 10^4$ (groups 3 and 6) cfu/mL of *C. jejuni* KC 40 (trial 2). Twenty-four h after inoculation the chicks were euthanized and the ceca as well as their contents were collected to examine the *C. jejuni* status of the animals (see below).

**Effect of the MCFA emulsion on *C. jejuni* transmission in broiler chicks**

Day-of-hatch broiler chicks (n = 60) were randomly assigned to six groups (n = 10 birds/group). Animals of groups 1 to 3 received standard drinking water, while those of groups 4 to 6 received water supplemented with 0.4% (vol/vol) MCFA emulsion (trial 3). All animals received a commercial starting feed *ad libitum*. The animals remained on the same feeding and drinking regime for the entire duration of the experiment. At 14 days of age, three out of ten birds per group were orally inoculated with ca. $3 \times 10^3$ cfu *C. jejuni* KC 40. Five days after inoculation, all birds were euthanized and the ceca as well as their contents were collected for *Campylobacter* enumeration (see below). Starting after inoculation, daily drinking water samples were taken from all groups and examined for the presence of *C. jejuni* bacteria.

**Determination of *Campylobacter* status and cecal counts**

Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with supplements. After homogenization, samples were serially diluted and 100 µL of each dilution was spread on mCCDA plates. After 24 h incubation at 42°C under microaerobic
conditions, plates were examined for the presence of specific *Campylobacter* colonies and/or enumerated. Bacterial counts in broiler drinking water (with or without the addition of 0.4% (vol/vol) MCFA emulsion) samples were analyzed analogously.

**Statistical analysis**

Data of all *in vivo* trials were analyzed by SPSS 17.0 software for Windows. The significance level $\alpha$ was set at 0.05. A Pearson chi-square test was performed to analyze the data of *in vivo* trial 2. For *in vivo* trials 1 and 3 *Campylobacter* counts were first transformed to log$_{10}$ counts before statistical analysis. Next, a one-way analysis of variance (ANOVA) was then carried out to compare the means of log$_{10}$-transformed counts in chicken cecal contents of all groups (MCFA-treated groups and control groups) in the *in vivo* trials. Significant differences were assessed by Bonferroni Post Hoc tests. *P*-values below 0.05 were considered significantly different.

**RESULTS AND DISCUSSION**

Recent research revealed marked anti-*Campylobacter* activity *in vitro* for the medium-chain fatty acids (MCFA) caproic, caprylic and capric acid (Hermans *et al.*, 2010). However, neither upon feeding these acids to broiler chicks, nor upon direct injection of their salt solutions into the ceca, *Campylobacter* counts were reduced. We therefore concluded that in-feed MCFA or their water-soluble salts are not capable of targeting *Campylobacter* colonization in chicks once the bacterium reaches the ceca. To assess whether water applications of MCFA might be more effective we performed a number of trials where a MCFA emulsion was administrated to the broiler drinking water. Such applications are more ergonomic and because drinking water of broilers is believed to play a key role in transmitting the bacterium through the flock, treating the water with MCFA might reduce *Campylobacter* transmission between birds of the same group (Chaveerach *et al.*, 2004; Messens *et al.*, 2009; Hermans *et al.*, 2011b). In this study we examined the anti-*Campylobacter* effects of a commercial MCFA-in-water (1/1) emulsion (containing caproic, caprylic, capric and lauric acid) at a 0.4% (vol/vol) dose. The influence of the emulsion on *Campylobacter* survival in treated drinking water and in the ceca of colonized birds was assessed. In addition, the effect on the susceptibility for *Campylobacter* colonization as well as on *Campylobacter* transmission between chicks was analyzed. Treating the drinking water used in these trials
with the MCFA emulsion at 0.4% (vol/vol) reduced the pH from 6.9 to 5.4, and thus the bactericidal potency of the acids (Hermans et al., 2010).

First, in trial 1, we assessed the effect of the MCFA emulsion on cecal Campylobacter counts of already colonized broiler chickens (figure 1.2.1). The results showed no significant ($P > 0.05$) differences in cecal bacterial counts of birds receiving treated or control water. These results are in line with our previous observations using in-feed coated MCFA. Other studies showed only a minor reduction in Campylobacter counts on cloacal swabs in therapeutic applications of monocabrin, while cecal Campylobacter counts were not significantly reduced. In addition, the spread of artificially infected to non-infected birds was not prevented (Hilmarsson et al., 2006). Metcalf et al. (2011) validated this observation, although in one trial a 3 log reduction in cecal counts was observed after therapeutic administration of 0.175% (wt/vol) caprylic acid to the water.

![Figure 1.2.1](image_url)

**Figure 1.2.1** Cecal Campylobacter counts of four-week-old broiler chickens ($n = 12$) inoculated with approximately $10^6$ cfu C. jejuni strain KC 40 at two weeks of age and receiving control drinking water (●) or drinking water supplemented with 0.4% MCFA emulsion (●). Bacterial numbers were log-transformed and represented as the mean ± standard deviation.

This raised the question whether MCFA are capable to reduce the susceptibility of and prevent Campylobacter colonization in chicks by exerting a bactericidal effect in the crop and stomach of the animals. For this purpose, in a next trial, we examined the effect of the MCFA emulsion on the susceptibility of individually housed two-week-old broilers for C. jejuni colonization. Birds were inoculated with C. jejuni doses of $3 \times 10^2 - 2 \times 10^4$ cfu per bird and
euthanized 24 h after inoculation. This time-frame allows *C. jejuni* to establish a high and stable colonizing population in the ceca of the chicks (Smith *et al.*, 2008; unpublished data). Our results, presented in table 1.2.1, show that less broilers were colonized with *Campylobacter* in groups receiving MCFA-supplemented drinking water compared to groups receiving standard drinking water. These results are in line with the observed increase in colonization threshold using in-feed MCFA applications (van Gerwe *et al.*, 2010). In our trial, a *C. jejuni* dose of $2 \times 10^3$ cfu was sufficient to colonize 60% of the birds receiving control water, while none of the ten birds receiving MCFA-supplemented drinking water were colonized in their ceca 24 h after inoculation. This statistically significant ($P = 0.03$) difference indicates that broilers receiving MCFA-supplemented drinking water from day-of-hatch onwards should be less prone to cecal colonization with *C. jejuni* compared to birds receiving un-supplemented water.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose <em>C. jejuni</em> KC 40</th>
<th>Number of colonized birds</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDW</td>
<td>-</td>
<td>0/4</td>
</tr>
<tr>
<td>CDW</td>
<td>$2.8 \times 10^3$</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>$2.2 \times 10^3$</td>
<td>6/10</td>
</tr>
<tr>
<td></td>
<td>$2.5 \times 10^4$</td>
<td>5/10</td>
</tr>
<tr>
<td>MSDW</td>
<td>$2.8 \times 10^2$</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>$2.2 \times 10^3$</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>$2.5 \times 10^4$</td>
<td>1/10</td>
</tr>
</tbody>
</table>

CDW = control drinking water; MSDW = medium-chain fatty acid-supplemented drinking water at a 0.4% (vol/vol) dose. Colonization status is represented as the number of *Campylobacter*-colonized birds out of 10 inoculated birds.

Table 1.2.1 Colonization status of 15-day-old broilers 24 hours after inoculation with *C. jejuni* strain KC 40

Finally, in trial 3 chicks were raised in six groups of ten birds each and were given either MCFA- or un-treated drinking water. Based on the results obtained in trial 2, three out of ten birds were orally inoculated with approximately $3 \times 10^3$ cfu of *C. jejuni*. Five days after inoculation of seeders all birds were euthanized and cecal bacterial counts were determined (figure 1.2.2). Differences observed between MCFA-treated and control repeats were statistically significant but small, due to a higher variability in bacterial counts in the MCFA-treated group (for individual values, see appendix A), the reason of which is unclear. However, it is clear that overall MCFA supplementation to drinking water did not result in an
overall significant \((P > 0.05)\) reduction in the cecal \textit{Campylobacter} load of these animals compared to birds receiving control water. This indicates that rapid bird-to-bird transmission mainly happened by the fecal-oral route, because no viable bacteria were recovered from the MCFA-treated water during the entire treatment period, while control water samples from all groups tested positive for \textit{Campylobacter} already two days after inoculation of the seeder birds (results not shown). \textit{In vitro} testing confirmed this observation (results not shown) with a 0.4\% (vol/vol) dose of the MCFA emulsion reducing \textit{C. jejuni} counts in drinking water to undetectable levels within the minute.

![Figure 1.2.2](image)

**Figure 1.2.2** Cecal \textit{Campylobacter} counts of twenty-day-old broiler chickens \((n = 10\text{ birds/group})\) receiving control drinking water (■) or drinking water supplemented with 0.4\% MCFA emulsion (●) from day-of-hatch until the end of the experiment. At 15 days of age, 3 out of 10 birds in each group were inoculated with approximately \(2.7 \times 10^3\) cfu of \textit{C. jejuni} KC 40. Bacterial numbers were log-transformed and represented as the mean ± standard deviation. The experiment was performed in triplicate. Columns with different letters (A, B and C) denote a statistically significant \((P < 0.05)\) difference in \textit{Campylobacter} load for two repeats from a different group (MCFA-treated or control).

Thus, on the one hand supplementing MCFA to the drinking water of broilers does neither reduce nor prevents cecal colonization of these animals with \textit{C. jejuni}. On the other hand, it prevents \textit{C. jejuni} survival in the drinking water and reduces the \textit{Campylobacter} colonization threshold of chicks. Probably, the antibacterial effect of the acids in the crop and stomach of the animals prevents the bacterium to reach and colonize the ceca. Indeed, Byrd \textit{et al.} (2001) observed reduced \textit{Campylobacter} counts in crops of broilers receiving drinking water
supplemented with 0.44% lactic acid. This indicates that supplementing MCFA to the drinking water of broiler chicken flocks throughout the whole rearing period, together with proper implementation of biosecurity measures (to limit Campylobacter exposure) could be helpful in preventing these flocks to become colonized. Indeed, Chaveerach et al. (2004) demonstrated a reduction in cecal Campylobacter counts of chicks receiving acidified drinking water only when birds were inoculated with a rather low inoculum concentration (~10^3 cfu). This strategy is likely more effective as a preventive measure for combating Campylobacter in poultry compared to administering MCFA after cecal colonization has already been established. Moreover, such an application would be more ergonomic compared to administering the acids to the feed, although also in-feed applications have been reported to be effective in reducing the probability of broilers to become colonized with C. jejuni (Skånseng et al., 2010, van Gerwe et al., 2010). However, the rapid killing of C. jejuni in MCFA-treated water at the applied doses at least excludes drinking water as a possible way for C. jejuni to enter and colonize a flock, an additional advantage over in-feed addition of MCFA. Indeed, drinking water equipment contaminated with C. jejuni is a possible contamination source for broiler chicks (Hermans et al., 2011b). Moreover, it has been found in longitudinal studies on commercial broiler flocks that C. jejuni is rapidly transmitted in the flock with drinking water as a vector ( Herman et al., 2003; Messens et al., 2009). In any case, it seems that the most effective way to benefit from the antibacterial effects of MCFA seems to be their preventive administration to reduce Campylobacter susceptibility of broilers and therefore the probability of Campylobacter to enter a flock, but further studies are needed to validate this hypothesis for three reasons. (1) In trial 2, birds were housed individually and only exposed to (inoculated with) Campylobacter once, while in commercially reared flocks birds are under a constant contamination pressure from the surrounding environment and each other (Hermans et al., 2011b). (2) At the end of trial 3, all birds were colonized with Campylobacter indicating that at least one seeder bird in all groups receiving MCFA-treated water was colonized and transmitted C. jejuni after inoculation with approximately 3 × 10^3 cfu of C. jejuni, although in trial 2 none of the ten birds orally inoculated with a similar dose (2 × 10^3 cfu) were colonized after one day. The reason for this discrepancy is not clear and can probably not solely be explained by the slightly higher inoculation dose used in trial 3. A more likely explanation is that C. jejuni transmission between the birds in trial 3 was responsible for a reduction in the apparent minimal infective dose (Conlan et al., 2011) determined in trial 2, because fecal Campylobacter shedding by colonized birds increases the contamination pressure for the remainder of the animals dramatically (Hermans et al., 2011b;
unpublished results). Under these conditions, MCFA are not capable anymore to prevent *Campylobacter* colonization of other animals. Therefore, it cannot be ruled out that MCFA supplementation in the drinking water is capable of reducing cecal colonization upon inoculating the seeders with lower inoculum doses. Also, although rather unlikely, it cannot be ruled out that birds negative for *Campylobacter* in their ceca 24 h after inoculation in trial 2 carried the bacterium more proximal in the gastro-intestinal (GI) tract. However, previous infection trials using this *C. jejuni* strain consistently resulted in marked cecal colonization at 24 hours post inoculation of both inoculated as well as sentinel chicks (unpublished results). Based on the results obtained, we suggest that MCFA supplementation induces a reduction in bacterial counts in the proximal GI tract of the animals, resulting in a bacterial population unable to reach and colonize the cecum within 24 hours post-exposure. (3) The degree of *Campylobacter* contamination pressure at which broiler flocks are exposed on the farm can only be speculated upon and depends on the farm that is studied. Therefore, large-scale field studies at broiler chicken farms are needed to determine at what level MCFA supplementation to the drinking water of commercially reared broiler chicks could contribute to the decreased probability of flocks to become colonized with *Campylobacter*.

To conclude, this study demonstrates that a water application of a MCFA emulsion might be successfully applied to prevent *Campylobacter* entrance into a broiler chicken flock by reducing the colonization probability of the birds and excluding drinking water as a potential contamination and transmission source.

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This work was financed by a grant of the Federal Public Service for Health, Food Chain Safety and Environment (FOD, Brussels, Belgium): project RT08/8-CAMPOUL. The excellent technical assistance of Hanne Vereecke and Nathalie Van Rysselberghe during laboratory analyses was greatly appreciated.

**References**


EFSA Panel on Biological Hazards (BIOHAZ). (2010b). Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. EFSA J, 8:2457 [107 pp.].


Individual cecal *Campylobacter* counts of twenty-day-old broiler chickens (n = 10 birds/group) receiving control drinking water or drinking water supplemented with 0.4% MCFA emulsion from day-of-hatch until the end of the experiment (results from *in vivo* trial 2). At 15 days of age, 3 out of 10 birds in each group were inoculated with approximately $2.7 \times 10^3$ cfu of *C. jejuni* KC 40. Bacterial numbers were log-transformed. The experiment was performed in triplicate.

<table>
<thead>
<tr>
<th>Control drinking water</th>
<th>Medium-chain fatty acid-treated drinking water</th>
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<tr>
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</table>

Underlined: seeder animals
† animal died before end of experiment
CHAPTER 3: EXPERIMENTAL STUDIES

3.2 THE CINNAMON-OIL INGREDIENT TRANS-CINNAMALDEHYDE FAILS TO TARGET CAMPYLOBACTER JEJUNI STRAIN KC 40 IN THE BROILER CHICKEN CECUM DESPITE MARKED ACTIVITY IN VITRO

D. Hermans, A. Martel, K. Van Deun, F. Van Immerseel, M. Heyndrickx, F. Haesebrouck and F. Pasmans

SUMMARY

*Campylobacter jejuni* is the most common bacterial cause of diarrheal disease in humans worldwide, with poultry products being a major source. Therefore, strategies to decrease *Campylobacter* colonization during primary production might aid in reducing the number of human campylobacteriosis cases. Several plant-derived compounds have been reported to possess anti-*Campylobacter* properties *in vitro*, so their use could be a promising candidate to reduce *Campylobacter* colonization in broiler chickens. To test this hypothesis, first a selection (caffeic, gallic, protocatechuic and vanillic acid, epigallocatechin gallate, trans-cinnamaldehyde and thymol) of plant-derived antimicrobial compounds (PDAC) was screened for anti-*Campylobacter* activity by determining minimal inhibitory concentrations (MIC) and setting up time-kill curves for *C. jejuni* strain KC 40. These experiments revealed marked antibacterial activity especially for the cinnamon oil ingredient *trans*-cinnamaldehyde (CIN). For this reason, this compound was tested in a broiler chick seeder model in which it was added to the feed in coated form at an effective concentration of 0.3% from day-of-hatch for the entire 22-day duration of the experiment. At 14 days of age, one out of three birds was inoculated with *C. jejuni* strain KC 40 and served as seeders. CIN was not able to reduce cecal *Campylobacter* colonization in this model which was confirmed in a cecal loop experiment. Despite CIN concentrations much higher than the MIC, *C. jejuni* numbers were not reduced compared to those in non-treated ceca at two and 24 hours after injection. In conclusion, this study shows a marked discrepancy between *in vitro* and *in vivo* activity of *trans*-cinnamaldehyde against *C. jejuni* strain KC 40.

**Key words:** plant-derived antimicrobial compound; *trans*-cinnamaldehyde; *Campylobacter jejuni*; broiler chicken cecum
INTRODUCTION

Today, Campylobacter infections are the leading cause of bacterial gastro-enteritis in many developed countries (Moore et al., 2005; EFSA, 2010b), with C. jejuni being the main causative agent (EFSA, 2009). Poultry and poultry products are considered a major source for human infection (Vellinga & Van Loock, 2002; Friedman et al., 2004; Wingstrand et al., 2006). It is estimated that chickens as a reservoir would account for up to 80% of human campylobacteriosis cases (EFSA, 2010a). Despite many efforts directed to minimize contamination of poultry with Campylobacter, the overall number of reported poultry meat consumption-related human campylobacteriosis cases has not been reduced (EFSA, 2009, 2010b).

Due to the high prevalence of Campylobacter in poultry, chicken meat is often contaminated with this pathogen. Campylobacter colonization of the chicken intestinal tract plays an important role in carcass contamination during slaughter (Herman et al., 2003; Rosenquist et al., 2003). Campylobacter is found abundantly in the poultry cecum (Corry & Atabay, 2001), so that small amounts of cecal contents can already remarkably increase the amount of Campylobacter on eviscerated broiler carcasses (Berrang et al., 2004). Two independent studies estimated that by lowering Campylobacter numbers on chicken carcasses 100-fold, the number of campylobacteriosis cases in humans associated with consumption of chicken meals could be reduced by 84% (Messens et al., 2007) and 97% (Rosenquist et al., 2003), respectively. Thus, measures taken to reduce Campylobacter colonization in the intestinal tract of poultry during primary production have potential for reducing contamination of poultry products, and thus the incidence of human campylobacteriosis. Despite many efforts, however, no such intervention measure is available to date (Lin, 2009).

Combating cecal Campylobacter colonization in poultry by supplementing plant-derived antimicrobial compounds (PDAC) to the feed could be promising. Many plant-derived substances have the ability to kill a wide range of bacteria in vitro, including Campylobacter (Cowan, 1999; Friedman et al., 2002). To date, however, only a limited number of them have been tested for their ability to combat cecal C. jejuni colonization of broiler chicks.

The first aim of this study was to screen a selection of PDAC on their in vitro activity against the highly colonizing C. jejuni strain KC 40 (Van Deun et al., 2008a; Hermans et al., 2010). This selection was based on reported anti-Campylobacter activity in vitro previously: caffeic, gallic and vanillic acid (Gañan et al., 2009), epigallocatechin gallate (Nagayama et al., 2002), trans-cinnamaldehyde (Johny et al., 2008), protocatechuic acid (Yin & Chao, 2008) and
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thymol (Anderson et al., 2009). The most active substances were tested further by setting up time-kill curves. The second objective was to evaluate the applicability of the most promising compound to reduce cecal Campylobacter colonization in broiler chickens.

MATERIALS AND METHODS

Experimental animals
Day-of-hatch Ross broiler chickens of both sexes from a local farm were raised in group until inoculation with Campylobacter and provided with feed and water ad libitum. Husbandry, euthanasia methods, experimental procedures and bio-safety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC numbers: 2009/114 and 2009/130). Chicks were examined for the presence of Campylobacter in mixed fecal samples. Samples were enriched in Nutrient Broth No.2 (NB2; Oxoid, Basingstoke, UK), supplemented with Modified Preston Campylobacter Selective Supplement (Oxoid) and Campylobacter-specific growth supplement (Oxoid). After 24 h incubation at 42°C under microaerobic conditions (5% O₂, 5% CO₂, 5% H₂, 85% N₂) enriched samples were plated on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid), supplemented with CCDA selective supplement (Oxoid) and Campylobacter-specific growth supplement, followed by microaerobic incubation at 42°C for 22 h. All mixed fecal samples proved to be Campylobacter-free prior to inoculation of chicks.

Bacterial strains and culture conditions
C. jejuni strain KC 40 from poultry origin was used in all experiments. Bacteria were routinely cultured for 17 h in NB2 with supplements at 42°C under microaerobic conditions (as above). C. jejuni bacteria were enumerated by preparing 10-fold dilutions in Hank’s Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on mCCDA, followed by microaerobic incubation at 42°C for 22 h.

Minimal inhibitory concentration of plant-derived antimicrobial compounds against Campylobacter jejuni
The minimal inhibitory concentration (MIC) of plant-derived antimicrobial compounds (PDAC) against C. jejuni was determined three times in triplicate at pH 6.0 using HCl or NaOH to obtain the desired pH-values. Pure caffeic, vanillic, protocatechuic and gallic acid, as well as epigallocatechin gallate, trans-cinnamaldehyde and thymol (all products from Sigma, St.
Louis, MO) were first dissolved in absolute ethanol (VWR International, France) and then added in serial two-fold dilutions in 96-well plates in Mueller-Hinton (MH) broth (Oxoid) ranging from 15.6 to 2000 µM (1000 µM for caffeic acid). These tested concentrations were chosen based on preliminary studies (data not shown). Test dilutions were prepared in such a manner that final ethanol concentrations in none of the wells exceeded 0.75% (vol/vol), the ethanol concentration at which growth-inhibitory effects start to occur toward C. jejuni KC 40 (data not shown). The inoculum was prepared by adding a C. jejuni suspension, grown for 17 h in NB2 with supplements, to sterile phosphate buffered saline (PBS) until a turbidity equivalent to a 0.5 McFarland standard, and diluting 1:5 in MH broth. Five µL of this suspension was inoculated into 195 µL medium in each well so that the final inoculum contained approximately $5 \times 10^5$ cfu/mL. These suspensions were incubated for 24 h at 42°C in a microaerobic environment (as above) after which microbial growth was assessed macroscopically: the lowest concentration completely inhibiting visible growth was considered the MIC.

**Time-kill curve experiments with selected PDAC**

Time-kill curve experiments were conducted in MH broth without (growth control) or with the addition of epigallocatechin gallate (EGCG) or trans-cinnamaldehyde (CIN) at 125, 250, and 500 µM. The solutions were brought to pH 6.0 using HCl or NaOH followed by inoculation with a C. jejuni KC 40 suspension grown for 17 h in NB2 with supplements, so the final bacterial concentration in these solutions was approximately $10^7$ cfu/mL. After inoculation, samples were kept at 42°C in a microaerobic environment and bacterial counts were determined at 0, 1, 2, 4, 8, and 24 h post-inoculation (pi).

**In vivo trial with supplementation of coated trans-cinnamaldehyde**

Day-of-hatch broiler chicks (n = 54) were randomly divided in six groups which were all housed separately. Birds of the first three groups were fed a standard feed and served as control birds. Chicks of the other three groups were fed the same standard feed, supplemented with 1% (wt/wt) coated cinnamaldehyde (cCIN; kindly provided by Koen Schwarzer, Nutri-Ad International, Kasterlee, Belgium), containing 30% (wt/wt) pure trans-cinnamaldehyde, so its final concentration in the feed was 0.3% (wt/wt), and 70% (wt/wt) coating material. Equal amounts of feed were provided for each group during treatment and care was taken that all animals had unlimited access to the feed. At the age of 15 days, three chicks of each group were orally inoculated with 1 ml of approximately $1.4 \times 10^8$ cfu of the highly colonizing C.
jejuni strain KC 40, allowing to cause infection in all animals of the same group (“seeder model”) (Van Deun et al., 2008a). At 21 days of age all chicks were euthanized by injection of T61 (Intervet, Belgium) in the wing vain (500 µL/chick), and the ceca as well as their contents were collected for C. jejuni enumeration (see below).

Cecal loop model
A cecal loop model was used to examine the effect of CIN on cecal Campylobacter numbers in broilers. This model allows direct comparison of C. jejuni numbers in both ceca of the same animal. One cecum (positive control) is injected with a control solution, while in the other a test compound is injected (Van Deun et al., 2008b; Hermans et al., 2010). Day-of-hatch broiler chicks (n = 9) were raised in group. At the age of 13 days, chicks were divided in three groups (n = 3) and housed in separate isolation units. At the age of 14 days, all chicks of groups 1 and 2 were inoculated with C. jejuni KC 40 (approximately 10^8 cfu (group 1), respectively 2 × 10^7 cfu (group 2)), while chicks of group 3 were kept in a Campylobacter-free environment. Cecal loops were applied, according to Hermans et al. (2010), at the age of 18 days (groups 1 and 3), or 21 days (group 2). Test solutions were prepared by dissolving CIN in absolute ethanol and diluting in HBSS until the desired concentration. One of the ceca of each animal of group 1 and 2 was injected with 200 µL 100 mM CIN in HBSS, while the other was injected with 200 µL HBSS with 2.67% (vol/vol) absolute ethanol, and served as control. In the animals of group 3, one cecum was injected with 100 µL 40 mM CIN in HBSS, the other with 100 µL HBSS with 1.07% (vol/vol) ethanol. After this, both ceca were injected with 100 µL of a C. jejuni KC 40 suspension of approximately 6.5 × 10^4 cfu/mL in HBSS.

After injection, the cecum was repositioned in the abdominal cavity and the peritoneum, muscles and skin were sutured. After two h (group 2) or 24 h (groups 1 and 3) the chickens were euthanized by injection with T61 and the ceca with their contents were collected for Campylobacter enumeration (see below).

Cecal Campylobacter jejuni enumeration
Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with supplements. After homogenization, a 10-fold dilution series was made in HBSS. Of each dilution, 100 µL was spread on mCCDA plates. After 22 h incubation at 42°C under microaerobic conditions, colonies were counted. For enrichment, diluted cecal samples in NB2 were incubated at 37°C under microaerobic conditions. After 24 h, samples were plated
on mCCDA and incubated at 42°C in a microaerobic environment. After 24 and 48 h plates were examined for the presence or absence of *C. jejuni*.

**Statistical analysis**

Data of the experiments were analyzed by SPSS 17.0 software for Windows. The significance level \( \alpha \) was set at 0.05. *Campylobacter* counts were first transformed to \( \log_{10} \) counts before statistical analysis. A one-way analysis of variance (ANOVA) was then carried out to compare the means of \( \log_{10} \) transformed counts in broth (time-kill curves) or in chicken cecal contents of all groups (treated groups and control groups) of the seeder model. Significant differences were assessed by Dunnet (time-kill curves) or Bonferroni (seeder model) Post Hoc tests. \( P \)-values below 0.01 (time-kill curves) or 0.05 (seeder model) were considered significantly different. A paired-samples \( t \)-test was performed to compare the means of \( \log_{10} \) transformed counts in chicken cecal contents of the treated group C and control group in the cecal loop experiment. \( P \)-values below 0.05 were considered significantly different.

**RESULTS**

**MIC of all tested PDAC against *C. jejuni* KC 40**

Table 2.1 shows the MIC values of the seven selected PDAC for poultry-derived *C. jejuni* strain KC 40 at pH 6.0. MIC value variation for each PDAC between the repeats was maximally one two-fold dilution. Epigallocatechine gallate (EGCG) and *trans*-cinnamaldehyde (CIN) had the lowest MIC value (125 \( \mu M \)) compared to all other compounds (\( \geq 1000 \mu M \)) for the tested strain.

<table>
<thead>
<tr>
<th>Compound</th>
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<tbody>
<tr>
<td>caffeic acid</td>
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<tr>
<td>vanillic acid</td>
<td>1000</td>
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<tr>
<td>gallic acid</td>
<td>2000</td>
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<tr>
<td>protocatechuic acid</td>
<td>&gt; 2000</td>
</tr>
<tr>
<td>epigallocatechin gallate</td>
<td>125</td>
</tr>
<tr>
<td><em>trans</em>-cinnamaldehyde</td>
<td>125</td>
</tr>
<tr>
<td>thymol</td>
<td>1000</td>
</tr>
</tbody>
</table>

*Table 2.1* Overview of the minimal inhibitory concentration (MIC) of selected plant-derived antimicrobial compounds, tested in Mueller-Hinton (MH) broth at pH 6.0 against *Campylobacter jejuni* strain KC 40. MIC values were determined three times in triplicate.
**Time-kill curves of selected PDAC against *C. jejuni* KC 40**

Based on the MIC values obtained in the previous experiment, time-kill curves were conducted to analyze the bactericidal effect of EGCG and CIN toward *C. jejuni* KC 40 at pH 6.0, using concentrations of 125, 250 and 500 µM over a 24-h period (figure 2.1). MH broth without acids and adjusted to pH 6.0 served as a growth control.

**Figure 2.1** Time-kill curves of *trans*-cinnamaldehyde (CIN, panel A) and epigallocatechin gallate (EGCG, panel B) at pH 6.0 against *Campylobacter jejuni* strain KC 40. Bacterial counts are represented as log_{10} CFU/mL. ♦, growth control; □, CIN or EGCG at 125 µM; ▲, CIN or EGCG at 250 µM; ○, CIN or EGCG at 500 µM. The experiment was performed in three independent trials and the mean results with standard deviation are shown. Significantly (*P* < 0.01) lowered bacterial counts compared with the control counts are denoted by an asterisk.
EGCG did not significantly ($P > 0.05$) reduce *C. jejuni* numbers (figure 2.1, B) at the concentrations tested. CIN was strongly bactericidal toward *C. jejuni* (figure 2.1, A) at all three concentrations tested. At 125 µM, it was initially growth-inhibitory (after 8 h post-inoculation (pi)) and lead to a significant ($P = 0.01$) reduction of the initial bacterial counts of over two logs after 24 h. CIN concentrations of 250 µM and 500 µM were strongly bactericidal within 8 h pi as shown by a significant ($P \leq 0.01$) decrease of > 4 log. CIN at 500 µM was already bactericidal within 4 h pi, as evidenced by significantly ($P \leq 0.05$) reduced (~3 log) bacterial counts compared with the control counts. Also after 24 h, both concentrations of CIN significantly ($P \leq 0.01$) reduced *C. jejuni* counts, which re-established to a level ca. three logs below the initial inoculum counts.

**In-feed cCIN does not reduce cecal colonization with *C. jejuni* in broiler chicks**

The three groups of 21-day-old broilers in the seeder model receiving feed supplemented with 0.3% (wt/wt) cCIN did not have significantly ($P > 0.05$) lower cecal *Campylobacter* counts, compared with the three groups receiving unsupplemented feed (figure 2.2). Feed intake (data not shown) was similar for all groups during the entire duration of the experiment.

**Figure 2.2** Average cecal *Campylobacter jejuni* counts in 21-day-old broilers (n = 9 chickens/group) fed unsupplemented feed or coated *trans*-cinnamaldehyde (cCIN) at 1% (wt/wt) for the entire duration of the experiment. Three chicks of each group were orally inoculated with approximately $1.4 \times 10^8$ cfu of *C. jejuni* strain KC 40 at 14 days of age and served as seeders. All birds were euthanized at 21 days of age. Values are represented as the mean of log$_{10}$ cfu/g cecal contents ± standard deviation (n = 9). The experiment was performed in triplicate.
CIN is not able to reduce *C. jejuni* numbers in the cecal loop model

The cecal content of 18- to 21-day-old broilers was estimated to be 1.0–2.0 mL. By injecting 100 µL of a 40 mM (as well as 100 µL *Campylobacter* suspension) or 200 µL of a 100 mM CIN solution, final concentrations in these ceca ranged from 1.8–3.3 mM or 9.1–17 mM. This was largely sufficient to cause a growth-inhibitory, bactericidal effect toward *C. jejuni* under *in vitro* conditions (see above). Final ethanol concentrations in all ceca were < 0.5% (vol/vol), thus beneath the growth-inhibitory level (see above). Despite these CIN concentrations, neither at 2 h nor at 24 h after injection mean *Campylobacter* levels in the cecal loops of broilers was reduced with our preset log_{10} 2, compared with the control cecum (figure 2.3).

![C. jejuni counts in cecal loops](image)

**Figure 2.3** *Campylobacter jejuni* counts in cecal loops of 19-day-old (n = 2 chickens/treatment) and 21-day-old (n = 3) broilers. In the left animal group, one cecum of a *Campylobacter*-free chick was injected with 100 µL of 40 mM *trans*-cinnamaldehyde (CIN), the other with 100 µL Hanks' Balanced Salt Solution (HBSS). Both ceca were injected with 100 µL of a 6.5 x 10^4 cfu/mL *C. jejuni* suspension and *C. jejuni* counts were determined 24 h later. In the middle and right animal group, one cecum of a *C. jejuni*-infected chick was injected with 200 µL 100 mM CIN, the other with 200 µL HBSS and *C. jejuni* counts were determined 24 h (middle) or 2 h (right) later. Values are represented as log_{10} cfu/g cecal contents.

**DISCUSSION**

The first objective of this study was to screen for the plant-derived antimicrobial compound (PDAC) with the highest *in vitro* activity against the highly colonizing *C. jejuni* strain KC 40
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out of a selection of substances already reported to possess marked anti-
Campylobacter activity in vitro. Minimal inhibitory concentrations (MIC) of seven PDAC toward C. jejuni were determined at pH 6. This pH approaches the mean cecal pH of broilers during their life-cycle (van der Wielen et al., 2000) and compensates for any pH-dependent effect. This approach revealed two compounds in particular to be very active against C. jejuni KC 40 with a MIC of 125 µM: the green tea polyphenol epigallocatechin gallate (EGCG) and the cinnamon oil ingredient trans-cinnamaldehyde (CIN). Time-kill curve experiments revealed bactericidal properties toward C. jejuni at pH 6 only for CIN at the tested concentrations: at 125 µM already, this compound was able to reduce bacterial counts after 24 h. However, bacterial counts tended to re-establish after an initial drop. Nevertheless, a marked bactericidal, dose-dependent activity of CIN toward C. jejuni in vitro was observed, as reported previously (Friedman et al., 2002; Johny et al., 2008; Ravishankar et al., 2008). Friedman et al. (2002) previously screened a more extensive selection of 23 essential oils and 96 oil compounds and found trans-cinnamaldehyde to be one of the most active substances toward C. jejuni in vitro. Hence, CIN seems a promising candidate to use as an in-feed PDAC for reducing cecal C. jejuni colonization of broiler chickens.

To our knowledge, the ability of CIN to reduce cecal Campylobacter colonization in broilers has not yet been examined. Therefore, our second objective was to analyze in the broiler cecum the anti-Campylobacter activity of CIN, classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Coating a compound on micro-beads is indispensable in a classic in vivo experiment where one administers the compound through the feed to allow it to reach the small intestine and the ceca (Van Immerseel et al., 2004), the latter representing the predominant site for Campylobacter colonization (Beery et al., 1988). Administration of micro-encapsulated CIN (cCIN) to the feed at 1% (wt/wt) was not shown to reduce feed uptake and was therefore chosen for the in vivo trial. Coating material consisted of fatty acids, mainly palmitic acid. When reaching the gut, CIN is released from the coating material by hydrolytic activity from the gut lipases. cCIN-supplemented feed, containing 0.3% (wt/wt) pure trans-cinnamaldehyde, did not reduce cecal Campylobacter colonization in 21-day-old chicks in our seeder model, where one on three birds was infected at 14 days of age. It is known that cinnamaldehyde is degraded very quickly in the digestive tract of piglets (Michiels et al., 2008). This might be extrapolated to the broiler gastro-intestinal (GI) tract and point to the utmost importance of developing a suitable coating material if one aims to obtain bactericidal CIN concentrations in more downstream areas such as the broiler cecum. It might thus be possible that the coating material used in this study was not capable of
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bypassing the premature degradation of CIN along the chicken GI tract leading to a lack of
effect in decreasing cecal *Campylobacter* levels in treated versus control groups. To examine
whether CIN is able to target *C. jejuni* in the broiler cecum, a cecal loop model, already
successfully applied in previous studies (Van Deun *et al.*, 2008b; Hermans *et al.*, 2010), was
used. Here, one directly injects the test compound into the ceca, making it unnecessary to coat
the compound. Twenty-four h after injection, CIN was not able to reduce *Campylobacter*
numbers in the cecal loops of broilers experimentally infected with *C. jejuni* strain KC 40 at
14 days of age or at the time of injection, despite concentrations much higher then the MIC.
To examine whether the lack of effect after 24 h was due to a re-establishment to original *C.
jejuni* counts after an initial drop, a group of animals was euthanized 2 h after CIN injection.
Also here, however, no reduction in cecal *Campylobacter* counts was observed.
Combined, these results suggest that *C. jejuni* is occupying a protected niche in the ceca of
broilers rendering them less susceptible to the bactericidal effects of CIN observed in vitro.
Also for capric acid we already showed that despite its marked anti-*Campylobacter* activity in
vitro, no reduction in *Campylobacter* counts in the broiler cecum could be obtained, both in a
cecal loop model as well as in a classic in vivo experiment (Hermans *et al.*, 2010). These
observations put the possible use of in-feed PDAC to combat cecal *Campylobacter*
colonization in poultry in question. To make clear, however, if the observed feature for capric
acid and CIN can be extrapolated to all PDAC, there is need for an in vitro fermentation
model mimicking the broiler’s cecal environment. Moreover, recent studies reported
observations that are not in line with our hypothesis. It was shown that the probability of the
bacterium to reach and colonize the broiler cecum can be reduced by supplementing medium-
chain fatty acids to the broiler feed (Solis de los Santos *et al.*, 2008a; van Gerwe *et al.*, 2009)
or even completely prevented with a combination of formic acid and sorbate (Skånseng *et al*.,
2010). Additionally, caprylic acid was shown to decrease cecal *Campylobacter* numbers by
multiple logs both in 15-day-old (Solis de los Santos *et al.*, 2008b) and market-aged (Solis de
los Santos *et al.*, 2009) broilers. It is clear that further research will have to elucidate the
potential contribution of in-feed PDAC to effectively combat cecal *Campylobacter*
colonization in chicks.
In conclusion, we here show a marked discrepancy between in vitro and in vivo activity of the
cinnamon-oil ingredient *trans*-cinnamaldehyde: neither in a classic in vivo trial nor in the
cecal loop experiment CIN was able to reduce cecal numbers of *C. jejuni* strain KC 40,
despite marked in vitro activity.
Acknowledgements

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3.3 PASSIVE IMMUNIZATION TO REDUCE *CAMPYLOBACTER JEJUNI* COLONIZATION AND TRANSMISSION IN BROILER CHICKENS
SUMMARY

*Campylobacter jejuni* is the most common cause of bacterial-mediated diarrheal disease worldwide. Poultry products are considered the most important source of *C. jejuni* infections in humans but to date no effective strategy exists to eradicate this zoonotic pathogen from poultry production. Here, the potential use of passive immunization to reduce *Campylobacter* colonization in broiler chicks was examined. For this purpose, laying hens were immunized with either a whole cell lysate or the hydrophobic protein fraction of *C. jejuni* and their eggs were collected. *In vitro* tests validated the induction of specific ImmunoglobulinY (IgY) against *C. jejuni* in the immunized hens egg yolks, in particular. In seeder experiments, preventive administration of hyperimmune egg yolk significantly (*P* < 0.01) reduced bacterial counts of seeder animals three days after oral inoculation with approximately 10^4 cfu *C. jejuni*, compared with control birds. Moreover, transmission to non-seeder birds was dramatically reduced (hydrophobic protein fraction) or even completely prevented (whole cell lysate). Purified IgY did not affect motility but promoted bacterial binding to intestinal mucus, suggesting enhanced mucosal clearance *in vivo*. Western Blot analysis followed by electrospray ionization mass spectrometry of in-gel digested, 2-dimensional separated proteins identified most of the immunodominant antigens of *C. jejuni*, which could be classified as involved in a variety of cell functions, including chemotaxis and adhesion. Some of these (AtpA, EF-Tu, GroEL and CtpA) are highly conserved proteins and could be promising targets for the development of subunit vaccines.

**Key words:** *Campylobacter jejuni*; chicken; passive immunization; hyperimmune egg yolk
INTRODUCTION

Today, *Campylobacter jejuni* is the most common cause of bacterial-mediated diarrheal disease in humans, worldwide (EFSA, 2011a). Despite many efforts aimed at minimizing *Campylobacter* contamination of poultry, no effective, reliable intervention measures exist to reduce bacterial numbers in the broiler gut (Hermans *et al.*, 2011a). In general, broiler chickens become colonized only at an age of two weeks (van Gerwe *et al.*, 2009). Transportation of immunoglobulin (Ig) Y, the major Ig class in chickens, from the hen to the embryo via egg yolk is believed to play a key role in the protection of young chicks with an immature immune system against *Campylobacter* colonization during two to three weeks post-hatch (Chalghoumi *et al.*, 2009a; Cawthraw & Newell, 2010). From two weeks onward, the concentration of maternally derived anti-*Campylobacter* IgY drops significantly, which coincides with an increased colonization susceptibility of the broiler chicks. Passive immunization of chicks may be prolonged by feeding broilers with high levels of anti-*Campylobacter* antibodies recovered from immunized hens (Chalghoumi *et al.*, 2009a). Following immunization, specific IgY is induced and transferred from the serum to the egg yolk (Chalghoumi *et al.*, 2009a), where it is accumulated at high levels (Dias da Silva & Tambourgi, 2010). Indeed, pre-incubating *C. jejuni* with IgY from immunized hens has previously been shown to reduce fecal *C. jejuni* counts in broilers experimentally inoculated with this mixture (Tsubokura *et al.*, 1997). In this study, also the effect of IgY on colonization in already-colonized animals was assessed. Results indicated that IgY induced only a limited therapeutic efficacy. After an initial drop, fecal *C. jejuni* numbers regained their original counts when treatment was stopped. Nevertheless, these observations indicate that IgY preparations from egg yolks could be a promising candidate to reduce *C. jejuni* colonization in broilers, but need to be optimized because no studies regarding this topic were reported since. Also, under farming conditions pre-incubation of the colonizing *C. jejuni* strains with IgY is not feasible. Moreover, in the study by Tsubokura *et al.* (1997) faecal bacterial counts were determined, which may not perfectly correlate to determining cecal counts, which is more sensitive (Woldemariam *et al.*, 2008). In the past two decades, some promising observations have been reported concerning the use of chicken egg yolk immunoglobulins for the prevention against other bacterial and viral pathogens as well, including amongst others streptococci (Smith *et al.*, 2001), *Helicobacter pylori* (Shimamoto *et al.*, 2002), human rotavirus, enterotoxigenic *Escherichia coli*, *Yersinia ruckeri* and *Salmonella* species (Mine &
Kovacs-Nolan, 2002; Chalghoumi et al., 2009a), using animal models or tested in humans. In these studies, protection was primarily observed after infection with the homologous strain. Passive immunization using yolk IgY thus seems a promising strategy to control *C. jejuni* colonization in broiler flocks. For this purpose, the first aim of this study was to examine whether artificial passive immunization by feeding experimentally inoculated broilers with hyperimmune eggs could reduce or prevent cecal *C. jejuni* colonization in broiler chicks. The second objective was to identify the *C. jejuni* antigens which were responsible for the induction of *C. jejuni*-specific IgY.

**MATERIALS AND METHODS**

**Experimental animals**

Commercial brown Leghorn laying hens and day-of-hatch Ross broiler chickens of both sexes from a local farm were raised in group until treatment. Birds were provided with a commercial feed and water *ad libitum*. Husbandry, euthanasia methods, experimental procedures and bio-safety precautions were approved by the Ethical Committee (EC) of the Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium (EC numbers: 2010/174 and 2011/029). Chicks were examined for the presence of *Campylobacter* in mixed fecal samples and proved to be free of *Campylobacter*.

**Bacterial strains and culture conditions**

*Campylobacter jejuni* strain KC 40 from poultry origin was used for all experiments. This strain colonizes chickens to a high level (Van Deun et al., 2008; Hermans et al., 2010; Hermans et al., 2011c). Bacteria were routinely cultured in Nutrient Broth No.2 (NB2, CM0067; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with Modified Preston *Campylobacter*-selective supplement (SR0204E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), at 42°C under microaerobic conditions (5% O<sub>2</sub>, 5% CO<sub>2</sub>, 5% H<sub>2</sub>, 85% N<sub>2</sub>). *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in Hank’s Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) and plating on modified charcoal cefoperazone deoxycholate agar (mCCDA, CM0739; Oxoid) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), followed by microaerobic incubation at 42°C for 22 h.
**Extraction of *C. jejuni* hydrophilic and hydrophobic proteins**

To prepare the immunizing agent, cells from a *C. jejuni* culture were collected by centrifugation (5000 × g for 30 min at 4°C), washed in HBSS and sonicated on ice for 30 seconds in extraction buffer 1 (EB1; 40 mM Tris, supplemented with tributylphosphine solution (Sigma), protease and phosphatase inhibitors, DNase and RNase) using a tip sonicator (Qsonica, Newtown, VS). After each sonication step, 12 in total, the sample was left on ice for 30 seconds. After centrifugation at 16,000 × g for 10 min at 4°C the supernatant (containing the hydrophilic proteins) was collected and the pellet resuspended in extraction buffer 2 (EB2; 40 mM Tris, supplemented with 5 mM urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% carrier ampholytes, 100 mM dithiothreitol and the respective inhibitors). After sonication and centrifugation of this suspension at 5,000 × g for 3 min to remove cell debris and unlysed cells, the supernatant (containing the hydrophobic proteins) was either pooled with the first supernatant (to obtain the whole cell protein lysate) or stored (4°C) separately.

**Immunization of laying hens**

Commercial Brown Leghorn chickens were randomly assigned to vaccination groups at the age of 19 weeks. Chicks were immunized with either the whole *C. jejuni* KC 40 cell lysate (EB1 + EB2) or 25 µg protein of the hydrophobic protein fraction only (EB2), by intramuscular injection of 250 µL of a 1:1 mixture with Freunds’ Complete Adjuvant at four different sites of the pectoral muscle. Chicks of the control group were immunized with a mixture of HBSS and Freunds’ Complete Adjuvants (FCA). Three booster immunizations using Freunds’ Incomplete Adjuvants (FIA) were given in a two-weekly time interval. Starting from one week after the second boost, eggs of the animals were collected and stored at 4°C.

**Determination of egg yolk and white IgY and IgA titers**

At weekly time intervals, starting from day 1, eggs were collected from both groups. The egg yolk and white were separated and pooled per group, diluted 1:5 (wt/vol) in distilled water and mixed thoroughly. After overnight incubation at 4°C the supernatant, containing the water-soluble fraction of the egg yolk and white, respectively, was collected for immunoglobulin quantification using enzyme-linked immunosorbent assay (ELISA). Wells of a 96 well Nunc-MaxiSorp microtiter plate were coated with 50 µL of a suspension of *C. jejuni* KC 40 cells (2 × 10⁷ cfu/mL) in coating buffer (2.16 g Na₂CO₃·10H₂O + 1.935 g NaHCO₃ in 500 mL H₂O) that were either heat-killed or processed to antigenic fractions by sonication.
Plates were incubated overnight at 4°C after which the wells were washed three times with HBSS followed by a final wash step using wash buffer (WB; PBS + 0.1% Tween 20). Next, the wells were incubated with 100 µL blocking buffer (BB; WB + 1% bovine serum albumin) at room temperature (RT) for 1 h to reduce non-specific binding of antibodies. Subsequently, 100 µL of doubling dilutions (in BB) of the supernatant was added to the wells in triplicate. The plates were incubated at RT for 90 minutes. After incubation the wells were washed three times using HBSS and once using WB and 100 µL of 1/10000 (in WB) of horseradish peroxidase (HRP)-labeled anti-chicken IgY (Sigma) or 1/20000 HRP-labeled goat anti-chicken IgA (Bio-Connect, The Netherlands) was added to each well. After incubation and washing as described above, 50 µL of 3,3’,5,5’-tetrathymethylbenzidine (TMB; Sigma) substrate was added. After 10 minutes the reaction was blocked by adding 50 µL 0.5 M H₂SO₄ to the wells. The absorbance was then measured at 450 nm using an automated spectrophotometer. Antibody titres from egg yolks/whites of immunized hens were reported as the highest dilution where the optical density (OD) was greater than the OD + three standard deviations of wells containing yolk/white originating from birds that only received adjuvans.

**Purification of IgY fraction from egg yolks**

IgY was purified from the hen eggs by the method of Bird & Thorpe (2002). Briefly, egg shells were broken and the yolk was separated from the white. Fifty mL of pooled yolk was placed on a filter paper in which a small whole was cut and pierced with a needle to loosen the membrane. Yolks were collected and diluted 1:5 (wt/vol) in Tris-buffered saline (TBS): 0.14 M NaCl in 10 mM Tris-HCl at pH 7.4. The solution was centrifuged at 2800 × g for 20 min at RT and the supernatant was collected. Subsequently, 120 µL dextran sulphate solution (10% in TBS) was added per 1 mL of supernatant and the solution was mixed well and incubated at RT for 30 min. Then, 50 µL of 1 M CaCl₂ was added per mL solution, mixed well and incubated for a further 30 min at RT after which the solution was centrifuged at 2800 × g for 30 min. The supernatant was collected and 20% (wt/vol) sodium sulphate was added while stirring. After complete dissolution, the solution was left standing for 30 min and centrifuged at 2800 × g for 20 min. The precipitate was redissolved in 10 mL TBS and centrifuged at 2800 × g for 20 min. The supernatant was collected and 8 mL 36% (w/v) sodium sulphate solution was added. The solution was left standing for 30 min at RT and centrifuged at 2800 × g for 20 min afterwards. The pellet was redissolved in 5 mL TBS. This solution was dialyzed against TBS and the purified immunoglobulin was filter sterilized.
Collection of broiler chicken intestinal mucus

Commercial and *Campylobacter*-free 14-d-old broiler chicks were euthanized and the small intestine was collected and gently rinsed with PBS to remove fecal material. The mucus was scraped from the mucosa with a glass slide covered in parafilm, diluted 1:3 with N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES, 25 mM, pH 7.4) and vortexed. The solution was centrifuged three times at 2000 × g for 15 min at 4°C. The supernatant containing the crude mucus was centrifuged two times more at 12,000 rpm for 15 min at 4°C and stored at -80°C. Protein content was determined using a Biorad protein assay kit (Biorad, Nazareth, Belgium).

Total protein content

Total protein content in mucus, IgY and *C. jejuni* lysate samples was determined using a Coomassie (Bradford) Protein Assay Kit according to the manufacturer’s instructions.

Mucus adhesion test

To examine the effect of IgY on the adherence of *C. jejuni* to intestinal mucus, bacteria were first incubated for 30 min at 42°C in HBSS only or supplemented with purified IgY from *C. jejuni*-immunized or HBSS-immunized hens. Mucus was diluted to a final concentration of 250 µg protein/mL in coating buffer. One hundred µL was immobilized per well of a maxisorb microtiter well plate and incubated overnight at 4°C. The wells were washed three times with HBSS and saturated with 1% (wt/vol) BSA in HBSS for 1 h at RT. After washing the wells two times with HBSS, 100 µL of the *C. jejuni*-IgY mixture was transferred to the wells. After incubation for 1 h at 42°C the wells were washed 15 times to remove unbound bacteria. Wells were thereafter treated with 200 µL 0.5% (vol/vol) Triton X-100 to desorb adherent bacteria and incubated for 30 min at RT while shaking. Next, 300 µL HBSS was added to each well and 10-fold dilutions of the wells were titrated on mCCDA plates.

Motility assay

To assess the influence of IgY on *C. jejuni* KC 40 motility, bacteria were pre-incubated with purified IgY and 20 µL was pipetted onto semi-solid Mueller-Hinton (MH) agar. After 24 h incubation at 42°C under microaerobic conditions, growth was assessed.
Effect of in-feed hyperimmune egg yolk on transmission of and cecal colonization with
*C. jejuni* in two-week-old broilers

In trial 1, day-of-hatch broiler chicks (n = 22) were raised in group. At 6 days of age the
chicks were randomly assigned to 2 groups (n = 11/group) and housed in separate isolating
chambers. Animals of group 1 were provided with feed containing 5% (wt/wt) egg yolk
(mixed manually through the feed) from hens immunized with HBSS/adjuvans, while birds of
group 2 were fed 5% (wt/wt) egg yolk from hens immunized with a whole cell *C. jejuni*
lysate/adjuvans mixture. Egg yolks were added to the feed for the remainder of the
experiment. Equal amounts of feed and drinking water were provided for each group during
treatment and care was taken that all animals had unlimited access to the feed and water. At
the age of 10 days, three chicks of both groups were orally inoculated with approximately 8 \times 10^3 cfu of *C. jejuni* strain KC 40. At 13 days of age all animals were euthanized (as described
above) and the ceca as well as their contents were collected for *C. jejuni* enumeration (see
below).

Trial 2 was performed analoguously. Day-of-hatch broiler chicks (n = 54) were raised in
group. At 6 days of age the chicks were randomly assigned to 6 groups (n = 9/group) and
housed in separate isolating chambers. Animals of groups 1, 2 and 3 were provided with feed
containing 5% (wt/wt) egg yolk from hens immunized with HBSS/adjuvans, while birds of
groups 4, 5 and 6 were fed 5% (wt/wt) egg yolk from hens immunized with the hydrophobic
protein fraction of *C. jejuni* mixed with adjuvans. At the age of 10 days, three chicks of each
group were orally inoculated with approximately 3 \times 10^4 cfu of *C. jejuni* strain KC 40.

Cecal *Campylobacter jejuni* enumeration

Ceca and contents were cut into little fragments, weighed, and diluted 1:9 (wt/vol) in NB2
with supplements. After homogenization, a 10-fold dilution series was made in HBSS. Of
each dilution, 100 µL was spread on mCCDA plates. After 22 h incubation at 42°C under
microaerobic conditions, colonies were counted. For enrichment, diluted cecal samples in
NB2 were incubated at 37°C under microaerobic conditions. After 24 h, samples were plated
on mCCDA and incubated at 42°C in a microaerobic environment. After 24 and 48 h plates
were examined for the presence or absence of *C. jejuni*.

Western blot analysis

To identify the immunodominant antigens of *C. jejuni* recognized by yolk IgY of immunized
hens, the whole *C. jejuni* lysate was separated by iso-electric focussing (pH 3-10), followed
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by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and 10% separating gel at 150V for 30 min and 200V for 60 min. The separated proteins were transferred electrophoretically (50V) onto nitrocellulose membranes (Bio-Rad) for 3 h. After washing with milliQ water, the membrane blots were blocked in 5% (wt/vol) milk in PBS for 1 h at RT. Subsequently, the blots were incubated overnight with purified egg yolk IgY, diluted to 1:5000 (vol/vol) in 5% (wt/vol) milk. After washing with 0.05% (vol/vol) Tween-20, the blots were incubated with horseradish peroxidase-conjugated goat-anti-chicken immunoglobulin G at a dilution of 1:50,000 in 5% (wt/vol) milk for 1 h. The blots were washed and proteins were immunodetected by enhanced chemiluminescence, using Supersignal West Dura Extended Duration Substrate (Pierce), and scanned and digitized using the Versa Doc imaging system. Blotting experiments were performed in duplo. To identify the immunodominant antigens during immunization of layer hens that may play a critical role in protective passive immunity in broiler chicks, the egg yolk IgY responses of HBSS-immunized and *C. jejuni*-immunized layers were compared by immunoblotting them with 2-dimensionally separated antigens. *C. jejuni* antigens reacting with hyperimmune egg yolk IgY were digested (see below) and sequenced (see below).

**In-gel digestion**

Spots of interest were cut into small gel pieces and washed twice in 25 mM ammoniumbicarbonate (ABC) with 50% acetonitril (ACN) for 10 min while shaking. Next, proteins were reduced in 10 mM dithiothreitol (DDT) with 25 mM ABC at 56°C, brought to RT and incubated further for 20 min. Alkylation was performed in 100 mM iodacetamide with 25 mM ABC for 45 min at RT and washed as describe above. Next, gel pieces were dehydrated in 100% ACN until they turned opaque and treated with trypsin (10 ng/µL) for 30 min on ice. After overnight incubation at 37°C, peptides were extracted in 50% ACN for 30 min. The samples were centrifuged at 15,000 × g and the supernatant was collected. The pellet was resuspended in 100% ACN, incubated for 30 min and centrifuged at 15,000 × g. The supernatant was collected and pooled with the first supernatant. Samples were dried and stored at -20°C.

**Mass spectrometric analysis**

*Liquid chromatography*

Prior to mass spectrometry the isolated peptides were separated on a U3000 nano-HPLC device (Dionex). Samples were first desalted on a pre-column (Acclaim PepMap 100 C18, 0.3
mm internal diameter (i.d.) × 5 mm, 5 µm particle size) at 20 µL/min with 0.1% formic acid for 10 min and subsequently separated on an analytical column (Pepmap C18 column 15 cm, particle size x µm, x mm internal diameter by 150 mm, Dionex). The gradient consisted of buffer A (0.1% formic acid (FA)) and buffer B (80% acetonitrile in 0.1% FA). The flow rate was 300 nL/min and the gradient elution was performed as followed: 30 min at 100% A, followed by a linear gradient of 4% B to 100% B over 70 min. Subsequently, an equilibration was performed for 10 min at 100% A. The column temperature was set at 60°C.

**Mass spectrometric analysis**

Identification of the peptides was performed on an electrospray ionization quadripole time of flight (ESI-QUAD-TOF) spectrometer (Ultima, Waters). Peptides were acquired at the range of 450 to 1650 m/z in MS. The seven most abundant multiple charges ions with a minimum intensity of 60 counts per second were subjected to MS/MS (between 50 and 2300 m/z). m/z ratios selected for MS/MS were excluded for 150 seconds.

Data analysis was performed against the *Campylobacter* protein databank from NCBI using the in-house search engine Mascot Daemon (2.3, Matrix Science, London, UK). An error tolerant search was performed with carbamidomethyl (C) as fixed modification. Deamidated (NQ) and oxidation (M) were set as variable modifications. Peptide mass tolerance and fragment mass tolerance were set at 0.35 Da and 0.6 Da, respectively. Maximum two miss cleavages were allowed. Proteins were only considered to be positively matched if the significance was below 0.05 (*p* ≤ 0.05) and at least one peptide passing the required bold red criteria from Mascot Daemon, indicating that at least one peptide had rank 1 with a significance below 0.05.

Uncentroiding for MS processing was performed at a peak half width of 0.2. The fitted model for MS peak picking was isotope distribution. The minimum signal to noise was set at 2 for MS as well as MS/MS.

**Statistical analysis**

Data of the *in vivo* trials were analyzed by SPSS 17.0 software for Windows. The significance level α was set at 0.05. *Campylobacter* counts were first transformed to log_{10} counts before statistical analysis. A one-way analysis of variance (ANOVA) was then carried out to compare the means of log_{10} transformed counts in chicken cecal contents of all groups (treated groups and control groups). Significant differences were assessed by Bonferroni Post Hoc tests. *P*-values below 0.05 were considered significantly different.
RESULTS

Determination of antibody titers from egg

In our study, C. jejuni-specific IgY was dramatically induced in egg yolk of hens immunized with a C. jejuni lysate, resulting in titers of up to 1:16,000 as determined by ELISA (results not shown). In contrast, C. jejuni-specific IgY in egg white and C. jejuni-specific IgA in egg white and yolk were not significantly induced after immunization and were present at only marginal concentrations in both groups.

In vitro anti-Campylobacter properties of egg yolk IgY

Pretreatment with purified IgY from yolks of C. jejuni-immunized hens significantly ($p < 0.05$) promoted bacterial binding of the homologous C. jejuni strain KC 40 to chicken intestinal mucus compared to IgY from HBSS-immunized (control) animals (figure 3.1). In contrast, C. jejuni-specific IgY did not affect C. jejuni KC 40 motility (data not shown).

![Figure 3.1](image)

Figure 3.1 Total adherent Campylobacter jejuni bacteria to chicken intestinal mucus after pre-incubation with HBSS (control, □) or IgY derived from C. jejuni-immunized (■) or HBSS-immunized hens (.rectangle). Values are represented as log cfu adherent bacteria/well. Statistical differences are denoted with one ($p < 0.05$) or two ($p < 0.01$) asterices.

In vivo anti-Campylobacter properties of egg yolk IgY

In in vivo trial 1, cecal C. jejuni numbers (figure 3.2) of inoculated broilers receiving hyper-immune egg yolks from whole cell C. jejuni lysate-immunized layers were reduced by > 5 logs ($p = 0.003$) compared to control broilers (3.3 ± 1.2 vs. 8.4 ± 0.6 (log$_{10}$ cfu/g cecal contents)). Moreover, transmission to non-seeder chicks was completely prevented, in
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contrast to the control group where non-seeder birds were all colonized at a similar \( (p = 0.143) \) degree to seeder chicks \( (7.3 \pm 1.1 \text{ vs. } 8.4 \pm 0.6) \). Together, this lead to a dramatic reduction \( (p < 0.001) \) in the overall cecal \textit{C. jejuni} count by \( \log_{10} 6.7 \) for birds receiving hyperimmune yolk \( (0.9 \pm 1.6 \log_{10} \text{ cfu/g cecal contents}) \) compared to birds receiving control yolks \( (7.6 \pm 1.1 \log_{10} \text{ cfu/g cecal contents}) \).

\[
\text{Figure 3.2} \quad \text{Individual cecal} \quad \textit{C. jejuni} \quad \text{counts of two-week-old co-housed broiler seeders (**) and sentinels (○) receiving standard feed supplemented with 5\% (wt/wt) egg yolk from either \textit{C. jejuni}- (left) or HBSS-immunized (right) layers, from day six until the end of the experiment. At 10 days of age, seeder animals were inoculated with approximately } 10^4 \text{ cfu of \textit{C. jejuni} KC 40. Values are represented as } \log_{10} \text{ cfu/g cecal content.}
\]

In \textit{in vivo} trial 2, which was performed in triplicate (data not shown), cecal \textit{C. jejuni} numbers of inoculated broilers receiving hyper-immune egg yolks from layers immunized with the hydrophobic protein extract of \textit{C. jejuni} were reduced by 2 logs \( (p = 0.002) \), compared to control animals \( (6.0 \pm 1.8 \text{ vs. } 8.0 \pm 0.4 \log_{10} \text{ cfu/g cecal contents}). \) Moreover, transmission to non-seeder chicks was dramatically \( (p < 0.001) \) reduced \( (1.5 \pm 2.5 \text{ vs. } 7.0 \pm 1.1 \log_{10} \text{ cfu/g cecal counts, averaged over all contact birds}). \) Together, this lead to a dramatic reduction \( (p < 0.001) \) in the overall cecal \textit{C. jejuni} count by \( \log_{10} 4.4 \) for broilers receiving hyperimmune yolk \( (3.0 \pm 3.2 \log_{10} \text{ cfu/g cecal contents}) \) compared to birds receiving control yolks \( (7.4 \pm 1.0 \log_{10} \text{ cfu/g cecal contents}). \)
Identification of immunodominant *C. jejuni* KC 40 antigens

Comparing Western Blots from 2-dimensional separated *C. jejuni* proteins immuno-stained with IgY from egg yolk of *C. jejuni* whole cell lysate-immunized laying hens (A) with those stained with IgY from HBSS-immunized hens (B) revealed several immunodominant antigens, specifically recognized by anti-*C. jejuni* KC 40 egg yolk IgY (figure 3.3).

![Figure 3.3](image)

**Figure 3.3** Western Blot of two-dimensionally separated *C. jejuni* KC 40 proteins, immunostained with purified immunoglobulin Y from egg yolks of laying hens immunized with *C. jejuni* KC 40 (A) or HBSS (B).

![Figure 3.4](image)

**Figure 3.4** Two-dimensionally separated *C. jejuni* KC 40 proteins. Proteins indicated with a number were sequenced by mass spectrometry.
Spots of interest on the Western Blot, immunostained with purified IgY from yolks of laying hens immunized with *C. jejuni*, were linked to the 2-dimensionally separated *C. jejuni* proteins on the gel (figure 3.4), digested in peptides and used for mass spectrometric (MS) analysis. For each spot, multiple proteins were identified. Proteins with a Mascot score of < 110 were excluded from the study. MS analysis identified several of the immunodominant antigens of *C. jejuni* KC 40. Results of duplicate experiments are represented in table 3.1 and table 3.2. Although in the second experiment not all protein spots could be identified, some additional differences were observed between the experiments: DnaK, glutamyl-tRNA synthetase, the hypothetical protein C8J_1250, oxidoreductase, 2-nitropropane dioxygenase, outer-membrane fibronectin-binding protein and a protein of the glutathionylspermidine synthase family protein were only found in the first experiment, while glycyl-tRNA synthetase alpha subunit, a probable thiol peroxidise, ribosomal protein L25, thioredoxin-disulfide reductase, the small unit of quinine-reactive Ni/Fe-hydrogenase, heat shock protein GrpE, hydrogenase expression/formation protein Hyp, dihydridipicolinate synthase and cysteine synthase were only detected in the second experiment. The reason for this discrepancy is not clear but could at least in part be explained by the rather low resolution obtained during isoelectric focusing using a pH gradient of pH 3-10. A gradient of pH 4-7 may lead to a better resolution, resulting in less contamination after in-gel digestion but possibly protein loss.

**Table 3.1** Predicted immunodominant *Campylobacter jejuni* antigens identified with HPLC-MS/MS (experiment 1)

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**Table 3.2** List of immunodominant *Campylobacter jejuni* antigens (experiment 2)

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<th>Arrow no.</th>
<th>Identified <em>C. jejuni</em> proteins</th>
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<th>Protein score</th>
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<td>Flagellin subunit FlaA</td>
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**DISCUSSION**

Immunizing laying hens and subsequently collecting their eggs is a cheap, straightforward and non-invasive method to obtain high amounts of specific antibodies (Mine & Kovacs-Nolan, 2002; Dias da Silva & Tambourgi, 2010). In our study, *C. jejuni*-specific IgY was dramatically induced in egg yolk of hens immunized with a *C. jejuni* lysate, resulting in titers of up to 1:16,000 as determined by ELISA (results not shown). In contrast, specific IgY in egg white and specific IgA in egg white and yolk were not significantly induced after
immunization and were present at only marginal concentrations in both groups. These observations are in line with those of Dohms et al. (1978), where transfer of radioactive labeled immunoglobulins (IgA, IgM and IgY) from the turkey hen into the egg was examined, indicating that only transfer of IgY to egg yolk is biologically relevant in the overall transfer of immunoglobulins into eggs.

In the first in vivo trial, using the whole cell lysate of *C. jejuni*, broiler chicks were provided with feed supplemented with (hyperimmune) egg yolk from day seven onward. At 10 days of age three out of 11 birds were orally inoculated with approximately $8 \times 10^3$ cfu *C. jejuni*. Colonization of these seeder animals is followed by transmission of *C. jejuni* to the remainder of the birds. Three days after inoculation, cecal *C. jejuni* numbers of seeder birds receiving hyperimmune egg yolk were significantly reduced compared to control seeders without IgY (by over 5 logs). Moreover, none of the non-seeder birds were colonized with *C. jejuni*, while chicks receiving control eggs carried high (ca. log$_{10}$ 7 cfu/g) bacterial numbers in their ceca. It is not clear at what site (the cecum or more proximal in the GI tract) the IgY fraction was active, but clearly it captured invading *C. jejuni* bacteria, disabling them to replicate and transmit to other birds. In addition, specific IgY increased bacterial binding of *C. jejuni* to chicken intestinal mucus, suggesting that it promotes bacterial uptake in the mucus layer for enhanced mucosal clearance. Because several of the immunodominant antigens were identified to be associated with the bacterial outer-membrane, a second (analogous) in vivo trial was performed using only the hydrophobic protein fraction of *C. jejuni*. Although the effects observed in this study were less pronounced compared to the results obtained with the whole cell lysate (probably due to the higher inoculation dose of the seeders ($3 \times 10^4$ cfu vs. $8 \times 10^3$ cfu)), hyperimmune yolks reduced cecal bacterial counts of seeders by 2 log and dramatically reduced *C. jejuni* transmission to contact birds. Previous passive immunization studies with *Salmonella* by other researchers indicated no effect at all on cecal colonization of broilers receiving feed supplemented with 5% (wt/wt) freeze-dried egg yolk powder containing anti-*Salmonella* spp. IgY (Chalghoumi et al., 2009b). These authors suggested that the antibodies were denatured and degraded along the GI tract, thereby reaching the ceca at insufficient concentrations. In our study, egg yolks were administered as such, because they contain antibacterial substances other than IgY and to exclude degradation during freeze-drying. Several reasons could explain the discrepancy between the two studies and include (1) bacterium-specific differences between *Salmonella* spp. and *C. jejuni* or differences in (2) IgY-formulations. Indeed, the reduction in cecal *C. jejuni* counts we obtained in our study could indicate that egg yolks form a protective matrix, allowing IgY to survive the digestive
enzymes and the low pH along the GI tract (Mine & Kovacs-Nolan, 2002); (3) experimental set-up, since in the study by Chalghoumi et al. (2009b) all birds were challenged with *Salmonella*, while we used a seeder experiment and age of the animals at challenge; and (4) immunization protocol, resulting in higher IgY titers in the hen egg yolks in our study.

Western Blot analysis followed by HPLC-MS/MS and BLAST analysis identified the most likely *C. jejuni* proteins responsible for the induction of specific IgY. Because a *C. jejuni* whole cell lysate was used, specific IgY could be induced against both membrane-bound as well as cytoplasmic proteins. Although our obtained results are fairly consistent with those reported in other studies examining antigenic proteins of *C. jejuni*, there was a striking absence in our study of Omp18, Cme, Cja and PEB proteins (Shoaf-Sweeney et al., 2008). These differences might be in part explained by *C. jejuni* strain differences. In addition, it is not clear to what extent repeated immunization of chickens in their breast muscle could lead to a differential immune response compared to that observed during (natural) commensal colonization with *C. jejuni*.

A first group of proteins that reacted with the purified egg yolk IgY are flagellar proteins: the flagellar hook protein FlgE and the major flagellin FlaA. Both proteins are known to be immunogenic and are needed for full motility of *C. jejuni* (Shoaf-Sweeney et al., 2008, Hermans et al., 2011b). FlgE mediates flagellar assembly and protein secretion in *C. jejuni* (Shoaf-Sweeney et al., 2008). In addition, mutation of the highly conserved FlaA results in a colonization defect (Wassenaar et al., 1993). These results are consistent with Shoaf-Sweeney et al. (2008) who reported the presence of maternal antibodies reacting with these flagellar proteins in the serum of laying hens. Despite these flagellar proteins were shown to be highly immunogenic in this study, specific IgY did not affect *C. jejuni* motility.

The major outer-membrane protein (MOMP) of *C. jejuni*, encoded by the *porA* gene, was identified in several gel pieces, pointing to its abundance in the *C. jejuni* proteome (Islam et al., 2010). In addition to being immunogenic (Huang et al., 2007), MOMP is involved in adhesion and transmembrane ion transport in *C. jejuni* (Islam et al., 2010). However, MOMP is extremely genetically diverse and several conformational epitopes have been implicated in the induction of protective immunity (Huang et al., 2007), thereby possibly hindering its use in vaccine applications.

An outer-membrane fibronectin-binding protein with an apparent mass of 36 kDa was presumed to be CadF (*Campylobacter* adhesion factor). CadF is a highly prevalent protein in *C. jejuni* where it is important for adhesion to chicken epithelial cells and cecal colonization.
of this host (Ashgar et al., 2007). This protein was, however, identified in only one of duplicate experiments with only few peptides matching, pointing to a contamination product. Methyl-accepting chemotaxis (MCP) proteins are transmembrane receptors for chemotactic stimuli (Hermans et al., 2011b). The C. jejuni genome encodes several MCP proteins and it is not clear which MCP protein, with an apparent mass of 41 kDa was identified in this study. Attached to these transmembrane receptors is the C. jejuni chemotaxis protein V (CheV), a coupling protein involved in transducing chemotactic signals in C. jejuni (Lertsethtakarn et al., 2011), which was also identified in this study.

Another immunodominant protein was the branched-chain amino acid ATP-binding cassette transport protein livJ, a periplasmic binding protein probably involved in transporting amino acids into the bacterial cell (Ribardo & Hendrixson, 2011) and crucial for chick colonization (Hermans et al., 2011b). An additional role for LivJ in the interaction of C. jejuni with the chick cecum was suggested by Ribardo & Hendrixson (2011).

ATP synthase is abundantly present and highly conserved among bacteria (Kovach et al., 2011). The protein is associated with the membrane where it performs its role in energy metabolism. In this study, the alpha subunit of ATP synthase F1 (AtpA) was found to be immunogenic in C. jejuni, as already reported for C. concisus (Kovach et al., 2011).

Most of the identified antigens in this study are proteins, mainly located in the bacterial cytoplasm where they perform their respective functions. Some of these antigens may nevertheless be interesting candidates for vaccine development.

GroEL and GrpE are heat shock proteins that play a crucial role in the C. jejuni stress response and the former has also previously been shown to be a immunodominant antigen of C. jejuni (Zhang et al., 2012). GroEL, belonging to the 60-kDa family, is an extremely conserved protein (Thies et al., 1999), and although mainly located in the bacterial cytoplasm, in Salmonella (and other bacteria) it is suggested to be expressed on the cell surface as well since GroEL has been shown to mediate Salmonella adhesion (Tsugawa et al., 2007).

The highly conserved translation elongation factor thermo unstable (EF-Tu) was found to be abundantly present in the C. jejuni lysate, which is in line with observations in other bacteria (Nieves et al., 2010). Despite its cytoplasmic role during protein synthesis, EF-Tu was shown to be translocated to the surface in several bacteria, where it mediates adhesion and invasion of host cells (Nieves et al., 2010). Immunization of mice with Burkholderia pseudomallei EF-Tu resulted in a potent immune response that was partially protective against melioidosis.
Finally, a putative secreted carboxyl-terminal protease of *C. jejuni*, CtpA, was identified. This protein has been shown to be highly prevalent and conserved in *Burkholderia mallei*, another Gram-negative bacterium (Bandara *et al.*, 2008).

Taken together, this study reports some very promising observations, but much more research is needed before a commercial product can be promoted. First of all, the observations from this study need to be validated using more animals. Second, it needs to be established whether this protective effect is maintained over periods exceeding three days post-inoculation. Also, the immunization protocol and the yolk dose in the feed need to be optimized. Further research will also have to elucidate whether this strategy is capable of providing cross-protection against heterologous *C. jejuni* strains. Eventually, this may lead to the development of a passive immunization strategy that can successfully control *C. jejuni* in chickens. If the relevant protective antigens of *C. jejuni* could be identified and studies regarding conservation and prevalence among *C. jejuni* strains are conducted, subunit vaccines able to target a wide range of *C. jejuni* strains could be developed for passive immunization applications.

To conclude, we here demonstrate for the first time that feeding broilers IgY-rich yolks from hens repeatedly immunized with *C. jejuni* (using either a whole cell lysate or with the hydrophobic protein fraction) through their feed dramatically reduces both *C. jejuni* numbers in the ceca after challenge with the homologous strain and transmission to non-inoculated contact birds, providing a solid base for further research regarding passive immunization to control *C. jejuni* colonization in broiler flocks.

**Acknowledgements**

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**References**


CHAPTER 4: GENERAL DISCUSSION
CHAPTER 4: GENERAL DISCUSSION

Gastro-intestinal illness caused by food-borne bacteria puts a serious public health burden worldwide, with *Campylobacter* spp. and *Salmonella enterica* as the zoonotic pathogens with the largest impact (EFSA, 2012). Recent research in this area resulted in the development of several intervention measures that can reduce the prevalence of these bacteria in food animals. Poultry very often is colonized with *Campylobacter* spp., but in contrast to some other bacteria (e.g. vaccination against *Salmonella*), no effective strategy exists to reduce the prevalence of this zoonotic pathogen in poultry production. As a consequence, *Campylobacter* is today the number one cause of bacterial-mediated gastro-intestinal disease in humans, with *C. jejuni* as the most important causative agent (Hermans *et al.*, 2011a).

*C. jejuni* is highly adapted to the chicken gut and colonized birds may carry a high bacterial load, especially in their ceca, without developing clinical signs. Broiler flocks are typically colonized from two weeks of age onward up to slaughter, resulting in high numbers of contaminated carcasses after processing (Hermans *et al.*, 2011b). Since the cecum is the only amplification point of *C. jejuni* throughout the entire food chain, on-farm intervention measures that reduce colonization of the ceca may have a significant impact on the number of campylobacteriosis cases in humans.

The general scientific aim of this PhD thesis was to develop an intervention measure that consistently reduces *C. jejuni* numbers in the ceca of broiler chickens by a thousand-fold, which would lead to a reduction in the number of human campylobacteriosis cases by 90%.

**Contribution of fatty acids to control *C. jejuni* in broiler chickens**

Concerns regarding antimicrobial resistance resulted in the ban of the use of antimicrobial agents in poultry as growth promoters by the European Union (Dibner & Richards, 2005). Therefore, other strategies will have to be developed for controlling *C. jejuni* in poultry.

For this purpose, the use of short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) (caproic, caprylic, capric and lauric acid) has been extensively studied. The antibacterial mode of action of these acids is not precisely defined. It is believed that acids diffuse into bacterial cells in the un-dissociated form and dissociate in the more alkaline bacterial cytoplasm. The excess in ions formed would eventually kill the bacterium by depleting ATP (Russel, 1992; Sun *et al.*, 1998). This might indeed explain the bactericidal, dose-dependent
activity of the tested MCFA observed at pH 6 in our study and the reduced sensitivity of acid-tolerant bacteria toward the antibacterial effects of organic acids (Van Immerseel et al., 2006), but does not explain why MCFA are more active compared to SCFA against several bacteria, including *C. jejuni* and *Salmonella* (Boyen et al., 2008). Probably, other factors such as composition of the backbone, hydrophobicity and the acid dissociation constant pKa of the acids will also determine the antimicrobial activity toward a given bacterium (Van Immerseel et al., 2006). Our observed MIC values for MCFA toward *C. jejuni* were markedly lower than those obtained for *Salmonella* (Boyen et al., 2008). However, while for *Salmonella enterica* serovar Enteritidis some reduction has been observed in cecal colonization of chicks fed caproic acid (Van Immerseel et al., 2004a), based on the results of this thesis it seems that MCFA administration to drinking water or feed is not capable to target *C. jejuni* in the chick cecum but can at most exert a bactericidal effect more proximal in the gastro-intestinal (GI) tract. For *Salmonella* Enteritidis, it was shown that MCFA reduce the expression of *hilA*, a key regulator of epithelial cell invasion, thereby possibly explaining the observed reduction in cecal *Salmonella* counts in chicks fed MCFA (Van Immerseel et al., 2004a). For *C. jejuni*, such events have not been identified.

*Medium-chain fatty acids are not effective in the chick cecum*

MCFA can be administered preventively to broiler flocks, from day-of-hatch, but more cost-efficient would be to incorporate them into the feed and/or drinking water prior to slaughter. Coating MCFA on micro-beads to prevent premature absorption and/or degradation of the acids in the upper GI tract is crucial in such applications, allowing them to reach the ceca (Van Immerseel et al., 2004b), which is the predominant site for *C. jejuni* colonization (Beery et al., 1988). However, coated and free MCFA-supplemented feed at 1% (wt/wt), as well as drinking water treated with a MCFA-in-water emulsion at a 0.4% (vol/vol) dose did not reduce cecal *C. jejuni* counts in experimentally inoculated chicks when administered three days before euthanization. Although it was not validated in these studies whether the active fatty acid concentration that reached the ceca was high enough for bactericidal activity, *in vitro* bactericidal concentrations did not prevent growth and survival when injected directly into the ceca. Therefore, developing proper coating materials to deliver larger acid amounts in the ceca seems useless. Alternatively, *C. jejuni* colonization in broilers could be prevented by administering MCFA from day-of-hatch up to slaughter. However, in seeder experiments, preventive administration of MCFA-treated drinking water did not reduce cecal *C. jejuni* counts of inoculated chicks. In addition, the spread of *C. jejuni* from experimentally
inoculated to non-inoculated birds was not prevented, in line with findings of Hilmarsson et al. (2006) and van Bunnik et al. (2012). Drinking water of broilers is believed to play a key role in C. jejuni transmission (Messens et al., 2009; Hermans et al., 2011b). Apparently, the reduced pH of the treated drinking water, which increased the bactericidal potency of the acids (Hermans et al., 2010) and even prevented the drinking water to become contaminated with C. jejuni, was not sufficient to prevent bird-to-bird transmission, which most likely happened by the fecal-oral route.

**Conflicting results**

Several contradictory observations to ours have been reported by others, suggesting that MCFA applications might be more effective in controlling C. jejuni in poultry than our results suggest. For instance, it was found that the ability of the bacterium to colonize the broiler cecum can be reduced by supplementing medium-chain fatty acids to the broiler feed from day-of-hatch (Solis de los Santos et al., 2008a) or even completely prevented with a combination of formic acid and sorbate (Skånseng et al., 2010). Additionally, therapeutic administration of caprylic acid to the feed decreased cecal Campylobacter numbers by multiple logs both in 15-day-old (Solis de los Santos et al., 2008b) and market-aged (Solis de los Santos et al., 2009) broilers. The reason for these contradictory observations is not clear, but may be explained by 1) differences in formulation of the acid in the feed and/or drinking water, allowing it to reach the ceca at higher concentrations in some studies, although our results with the cecal loop model suggest that MCFA are not effective in targeting C. jejuni in the cecum even at elevated concentrations; 2) C. jejuni strain-specific properties, as we inoculated the chicks with a highly invasive strain that might be less susceptible toward the effects of MCFA in the broiler cecum compared to less invasive strains; or 3) other factors, such as differences in genetic background of the birds used or differing dietary influences on cecal biochemistry.

**Protective niche theory**

Our findings made us conclude that MCFA can neither target C. jejuni in the chick cecum, nor prevent bird-to-bird transmission with C. jejuni after the first bird is colonized. Thus, the use of fatty acids will most likely not result in either a substantial decrease in the number of C. jejuni-colonized animals or the number of viable bacteria in the ceca of colonized animals. Apparently, C. jejuni harbours a “protective niche”, rendering the bacteria unresponsive to the bactericidal effect of organic acids observed in vitro. One of the major questions remaining to
be answered is why there is such a big discrepancy between *in vitro* and *in vivo* observations for the tested compounds. After all, the acid concentrations needed to inhibit growth of or to kill *C. jejuni* are approximately 10-fold lower compared to other bacterial pathogens, such as *Salmonella enterica*. In contrast, *in vivo* concentrations that are higher compared to those successfully applied to moderately reduce *Salmonella enterica* serovar Enteritidis numbers in chicks, failed to induce any effect at all against *C. jejuni*. Intestinal mucus was shown to reduce the anti-*C. jejuni* activity of both SCFA (Van Deun *et al*., 2008) and MCFA, indicating that the chicken intestinal mucus layer, which covers the entire surface of the chicken intestinal tract (Forstner *et al*., 1995), clearly plays a protective role. Possibly, it forms a physical barrier between the bacterium and the acid, because *C. jejuni* is found deep in the cecal crypts within the mucus layer (Beery *et al*., 1988). Thus, whereas the chicken intestinal mucus layer usually is regarded as part of the innate host defense system and thus an anti-bacterial barrier (Forstner *et al*., 1995), it seems that in the case of *C. jejuni* it forms a protective and supportive environment making it hard to eradicate this bacterium from the chicken intestinal tract, once colonized. Although some chicken intestinal mucus factors promoting *C. jejuni* colonization have been identified (such as providing all necessary nutrients for optimal growth, promoting chemotaxis and enhanced secretion of the *Campylobacter* invasion antigens), it is not clear how the intestinal mucus layer contributes to increased resistance of *C. jejuni* against fatty acids.

**Reducing the colonization threshold**

This raises the question whether MCFA are capable of exerting a bactericidal effect in more proximal sites of the GI tract, thereby possibly preventing cecal *C. jejuni* colonization in chicks. Indeed, we demonstrated that MCFA-treated drinking water at a 0.4% (vol/vol) dose from day-of-hatch increased the colonization threshold of individually housed broilers when inoculated with low (2.8 × 10^2 cfu) to moderate (2.2 × 10^3 cfu) *C. jejuni* numbers, which is in line with Chaveerach *et al*., (2004) and with observations made using an in-feed approach (van Gerwe *et al*., 2010). MCFA-treatment reduced the pH of the drinking water from neutral to 5.4, thereby increasing the bactericidal potency of the acids. This reduced pH might aid in killing *C. jejuni* in the crop and stomach, thereby preventing a bacterial population to reach and colonize the cecum. Indeed, Byrd *et al*., (2001) observed reduced *C. jejuni* counts in crops of broilers receiving drinking water supplemented with 0.44% lactic acid. Again, however, in our study, MCFA-treated drinking water did not result in an overall reduced cecal *C. jejuni* load of co-housed chicks, five days after inoculation of seeders with this moderate (3 × 10^3
cfu) *C. jejuni* dose. Possibly, *C. jejuni* transmission between birds was responsible for a reduction in the apparent minimal infective dose (Conlan *et al*., 2011), because fecal *C. jejuni* shedding by colonized birds increases the contamination pressure for the remainder of the animals dramatically (Hermans *et al*., 2011b). Under these conditions, MCFA are not capable anymore to prevent *C. jejuni* colonization of other animals. It can, however, not be ruled out that MCFA are capable of preventing cecal colonization of co-housed birds upon inoculating seeders with lower *C. jejuni* numbers.

**Conclusion**

Although not effective in the ceca, MCFA prevent *C. jejuni* survival in treated drinking water and may kill *C. jejuni* to some degree in more proximal sites of the GI tract. On-farm, however, birds are under a constant contamination pressure from the outside environment and each other. Therefore, bacteria that survive and reach the ceca may eventually result in a colonized broiler, acting as a transmission source after a stable colonizing population has been reached in the ceca. The rapid fecal-oral spread after the first bird is colonized will then eventually result in the whole flock being colonized at slaughter age. At this point, it seems that the use of MCFA is useless.

Nevertheless, supplementing MCFA to the drinking water of broiler chicken flocks throughout the whole rearing period, together with proper implementation of biosecurity measures (to limit *C. jejuni* exposure) could be helpful in preventing these flocks to become colonized. Moreover, because drinking water and drinking water equipment contaminated with *C. jejuni* contribute to the rapid transmission of *C. jejuni* in a broiler flock (Messens *et al*., 2009; Hermans *et al*., 2011b), the rapid killing of *C. jejuni* in MCFA-treated water at the applied dose inhibits *C. jejuni* transmission via the drinking water, and excludes drinking water as a possible way for *C. jejuni* to enter and colonize a flock, which are additional advantages over in-feed addition of MCFA. In addition, in a very recent study, it was demonstrated that MCFA administration significantly reduced indirect transmission between spatially separated chicks (van Bunnik *et al*., 2012), suggesting that drinking water acidification may be capable in reducing between-flock transmission. In any case, it seems that the most effective way to benefit from the antibacterial effects of MCFA is their preventive administration to increase the number of viable *C. jejuni* bacteria needed for successful colonization of broilers and therefore the probability of *C. jejuni* to enter a flock. Large-scale field trials are needed to examine whether such a reduced colonization threshold,
imparted by treating the drinking water with MCFA, is capable to prevent cecal *C. jejuni* colonization in chicks under farming conditions.

**Contribution of plant-derived antimicrobial compounds to control *C. jejuni* in broiler chickens**

Screening experiments of several plant-derived compounds were performed at pH 6, approaching the mean cecal pH of broilers during their life-cycle (van der Wielen et al., 2000), which compensates for any pH-dependent effect. Among the plant-derived antimicrobial compounds (PDAC) tested, the cinnamon-oil ingredient *trans*-cinnamaldehyde (CIN) and the green tea polyphenol epigallocatechin gallate (EGCG) were the most effective (MIC of 125 µM) at preventing *C. jejuni* growth. In addition, for CIN a lower minimal bactericidal concentration was observed compared to EGCG. Also Friedman et al. (2002) found *trans*-cinnamaldehyde to be one of the most active substances toward *C. jejuni* in vitro out of a more extensive selection of compounds. Hence, CIN seemed a very promising candidate as a feed additive to reduce *C. jejuni* colonization in poultry and was therefore tested *in vivo*. Using a seeder model, we showed that CIN is not able to prevent transmission and colonization and does not reduce cecal *C. jejuni* counts at the concentrations tested, even when the compound was micro-encapsulated. *In vitro* incubation of *C. jejuni* with CIN at doses of 250 µM or higher reduced bacterial counts below the detection limit within eight hours post-inoculation, but viable bacteria were detected 24 hours pi. The reason for this re-emergence is not clear, but might possibly be explained by the development of a resistant subpopulation of *C. jejuni* (although unlikely after such short incubation times), or the ability of *C. jejuni* to metabolize or degrade *trans*-cinnamaldehyde. In addition, in pigs, CIN is degraded very quickly along the GI tract (Michiels et al., 2008). Whether this is also the case in chicks is not clear, but at least some of these events may possibly explain the ineffectivity of CIN *in vivo*. However, direct injection in chicken ceca of CIN concentration of multiple times the MIC did not reduce *C. jejuni* counts two or 24 hours after injection, thereby excluding all these possibilities and suggesting a rather similar reason for not being effective as observed for fatty acids.
Influence of the model and \textit{C. jejuni} strain used on experimental outcome

For all \textit{in vivo} trials, a highly invasive and efficiently colonizing \textit{C. jejuni} strain (\textit{C. jejuni} KC 40, isolated from chicken feces and passaged twice in broilers) was used, resulting in a highly reproducible model in which all animals were colonized, with very high cecal bacterial counts, 24 hours after inoculation. Given these characteristics of \textit{C. jejuni} KC 40, it could be possible that inoculation with this strain is too overwhelming for allowing any potentially beneficial effect to be noticed. However, preliminary experiments using a low invasive \textit{C. jejuni} strain (KC 59.1) revealed similar (even slightly higher) colonization levels with the absence of a positive effect when using a mixture of MCFA (results not shown). Moreover, because control measures should be able to target most, if not all, \textit{C. jejuni} strains, the choice for working with \textit{C. jejuni} KC 40 is definitely warranted.

Second, on-farm birds are under a constant contamination pressure from the surrounding environment, while in our models birds were challenged with \textit{C. jejuni} only once. On the other hand, in some models, (all) animals were inoculated with high doses of the \textit{C. jejuni} strain. It is doubtful that such events will take place on-farm. However, based on the literature and experiments conducted at this laboratory it seems that \textit{C. jejuni} will be transmitted to all animals shortly after the first animal is colonized, even if only a few birds are challenged with relatively low \textit{C. jejuni} doses. Indeed, it was estimated that all (20,000) chicks in a flock will be colonized seven days after \textit{C. jejuni} is detected in the first bird (Stern \textit{et al.}, 2001). Therefore, it is doubtful that less aggressive models could have led to more satisfactory results and the absence of effectiveness is probably not attributable to the models used. The marked reduction in cecal \textit{C. jejuni} counts (over three logs) obtained in our therapeutic model using the macrolide tylosin definitely supports this hypothesis.

Urgent need for alternative strategies

Due to practical concerns, only a limited number of fatty acids and plant-derived antimicrobials were tested \textit{in vivo}. An \textit{in vitro} fermentation model mimicking the broiler’s cecal environment could be helpful to examine whether our observations with the studied compounds can be extrapolated to all fatty acids and PDAC. Nevertheless, from our observations it is clear that caproic acid, caprylic acid, capric acid and trans-cinnamaldehyde at relevant doses are not capable to consistently reduce the cecal \textit{C. jejuni} load of broiler
chickens by at least three logs. Therefore, it has to be assessed whether other on-farm intervention measures are capable of reaching this goal.

For this purpose, several strategies could be used, including the use of bacteriophages, competitive exclusion, bacteriocins, vaccination and passive immunization, as summarized by Hermans et al. (2011a).

The use of bacteriophages seems very promising but has the disadvantage of being strain-specific. Therefore, the reducing potential of a specific phage will differ from one region to another depending on the prevalent C. jejuni strains. Although the problem of this specificity can be reduced by using a bacteriophage cocktail, the development of a universal bacteriophage product seems not feasible. In addition, a relatively high degree of bacteriophage resistance has been reported with this strategy. However, bacteriophage-resistant phenotypes are poor colonizers and in absence of selective (bacteriophage) pressure, they regain their efficient colonization and phage-sensitive status (Scott et al., 2007).

Competitive exclusion (CE) cultures could provide the ideal intervention measure if they share the same colonization site as C. jejuni. By blocking interaction/colonization sites and competing for essential nutrients they could prevent C. jejuni from colonizing their protective cecal niche. However, the reported observations using CE are not very promising (Hermans et al., 2011a) and more research is needed regarding both the chicken and C. jejuni factors involved in this interaction. This may substantially reduce the number of relevant candidates, eventually leading to the identification of CE cultures effective in preventing C. jejuni from colonizing its cecal niche. Bearing in mind our “cecal niche theory”, such an approach could render C. jejuni more susceptible to the antibacterial properties of organic acids and PDAC, making their potential contribution to C. jejuni control in chicks not so limited after all. To test this hypothesis, studies regarding the simultaneous use of CE cultures (to prevent C. jejuni colonization) and organic acids or PDAC (to enhance mucosal killing of C. jejuni), are needed.

The use of purified bacteriocin from bacteriocin-producing Enterococcus isolates seems very promising. Svetoch & Stern (2010) reported dramatic reductions by over 6 logs in cecal C. jejuni counts. However, there is a lack of knowledge concerning potential development of resistance to bacteriocins in C. jejuni. Although in vitro emergence of bacteriocin-resistance in C. jejuni was recently shown to be rather limited, it is currently not known whether high selection pressure could promote bacteriocin emergence in vivo (Hoang et al., 2011).
Finally, also immunization-based approaches should be considered for *C. jejuni* control. Although in chickens *C. jejuni* is able to invade epithelial cells and colonize the spleen (Van Deun *et al.*, 2008), its interaction with this host is believed to be of commensal nature, devoid of any inflammatory response of the chicken intestinal wall and clinical signs during colonization (Hermans *et al.*, 2011c). Therefore, direct vaccination of young broiler chickens is not expected to induce a potent immune response, although some reduction (log$_{10}$ 1.4 cfu/g) has been observed in cecal *C. jejuni* numbers of birds vaccinated with a *Salmonella* Typhimurium mutant expressing CjaA (Buckley *et al.*, 2010). In addition, manual vaccination of all broiler chicks in a flock, *per os* and definitely through injection, would be very time-consuming. Alternatively, as often performed for vaccinating breeder chickens against *Salmonella*, the vaccine could be administered via the drinking water. To our knowledge, however, no reports have been published where such an approach was successful in reducing cecal *C. jejuni* counts in broiler chicks.

**Passive immunization: a promising alternative approach?**

The negative features of direct vaccination (lack of an appropriate immune response and cost-effectiveness) might be circumvented by hyper-immunizing laying hens and subsequently collecting their eggs. The induced immunoglobulins (mainly IgY) are transported to and accumulated in the egg yolks of these animals. By simply collecting these IgY-rich eggs and administering the yolks to the feed of broiler chickens, *C. jejuni* colonization may be prevented or reduced. Passive immunization thus seems very promising and was therefore evaluated as a possible alternative to in-feed antibiotics to combat *C. jejuni* colonization in chickens.

Passive immunization has been shown to be effective against a wide variety of disease-causing bacteria, including *H. pylori* (Shimamoto *et al.*, 2002). In a study from 1997 (Tsubokura *et al.*, 1997), it was shown that passive immunization might also have a beneficial contribution to reduce *C. jejuni* shedding in poultry. However, this is the only published report concerning this matter and this technique needs optimization.

For this purpose, during this thesis, commercial laying hens were repeatedly immunized with, either a whole cell lysate or the hydrophobic protein fraction of, *C. jejuni* KC 40, using Freund’s Complete (first immunization) and Incomplete (booster immunizations) Adjuvans for inducing a potent immune response. Five weeks after the first immunization, egg yolks started to accumulate high titers of specific IgY, as judged by enzyme-linked immunosorbent
assay, which were maintained for several weeks (monitored up to the end of the *in vivo* trial). Administering these egg yolks to the feed of chicks at 5% (wt/wt) tremendously lowered cecal *C. jejuni* colonization of seeder animals upon homologous inoculation and (almost) completely prevented transmission to contact birds. *In vitro* analyses showed that purified IgY promoted *C. jejuni* binding to intestinal mucus, which may be responsible for increased mucosal clearance of *C. jejuni* bacteria. In contrast, IgY did not alter motility of the homologous *C. jejuni* strain at these concentrations, which is rather unexpected given the high immunogenicity of *C. jejuni* flagella (Sahin *et al.*, 2001; Shoaf-Sweeney *et al.*, 2008). In addition, Shoaf-Sweeney *et al.* (2008) did show reduced motility of a homologous, but not that of a heterologous strain by maternal antibodies *in vitro*.

Two-dimensional gel electrophoresis followed by Western Blot analysis and electro-spray ionization-mass spectrometry identified several of the immunodominant antigens of *C. jejuni* KC 40, including the flagellar hook protein FlgE2. The immunodominance of this flagellin protein is in line with the reported immunogenicity of the *C. jejuni* flagellum (Sahin *et al.*, 2001; Shoaf-Sweeney *et al.*, 2008) but raises further questions why purified IgY did not affect *C. jejuni* KC 40 motility in our *in vitro* experiments.

**Conclusions and what to do in the mean time?**

To conclude, although some on-farm approaches seem promising and deserve further attention, there is still no effective and reliable commercial intervention measure available to date. As a consequence, the number of human campylobacteriosis cases remains strikingly high (EFSA, 2012).

The results of this thesis indicate that especially the use of passive immunization is very promising to reduce *C. jejuni* colonization in poultry and should deserve full further attention. We suggest that recombinant production of conserved immunodominant antigens, followed by studying them in passive immunization experiments could lead to the identification of *C. jejuni* antigens crucial for chick colonization. Promising candidates for this purpose may include GroEL, EF-Tu, AtpA, CtpA and MCPs. Increased knowledge on the interaction of *C. jejuni* with its chicken host could then be exploited for further fine-tuning of passive immunization as an effective intervention measure to control *C. jejuni* in poultry. Protective antigens could be used to hyperimmunize chicks, collecting their eggs and feeding them to broilers. Such an approach allows to pool eggs from different hens and/or from one hen at
different time intervals, thereby reducing batch-to-batch variation. However, more research is needed to validate whether such an approach would be feasible on a large scale. Alternatively, antibodies against these protective antigens, if any, could be identified and recombinantly and cost-efficiently produced in plants, which can be mixed through the chicken feed. However, much more research is needed to determine whether such an approach is realistic.

It is not clear whether the current approach of preventively feeding broilers with 5% (wt/wt) egg yolk would be cost-effective. And if so, to what extent would farmers be willing to pay the price to prevent their flocks from colonization with a commensal bacterium that does not lead to economical losses, and as such does not give them any advantage? This urges the need for a kind of “bonus system” where farmers are encouraged to consistently deliver _Campylobacter_-free batches.

In the meantime, it is of utmost importance to inform the customer about the risk posed by _Campylobacter_-contaminated chicken meat products and to enhance biosecurity measures on the farm and during slaughter. Proper implementation of on-farm measures, such as the use of a footbath before entering the broiler house, cleaning and disinfection of farm equipment and insect and rodent control programs should be able to reduce the prevalence of _C. jejuni_ in broiler flocks, while proper cleaning and disinfecting of abattoir equipment should reduce the likelihood for cross-contamination. Such measures can be readily and rather cost-effectively be implemented and should be aimed to keep the number of _Campylobacter_-contaminated chicken meat products under control. Possibly, these measures could be combined with treating the drinking water of broiler flocks with medium-chain fatty acids to increase their _C. jejuni_ colonization threshold, but large-scale field trial are needed to prove the effectivity of this approach. It is very doubtful, however, that these measures alone would be sufficient to dramatically reduce the _C. jejuni_ prevalence in poultry.
References


General discussion


SUMMARY
SUMMARY

Campylobacter jejuni is the most common cause of bacterial-mediated diarrheal disease in humans worldwide and poses a serious health burden. Poultry is considered the most important source for campylobacteriosis in humans. C. jejuni is highly adapted to the chicken gut and colonized birds carry a very high bacterial load, especially in their ceca, without developing any clinical signs. Broiler flocks are typically colonized from two weeks onward up to slaughter, resulting in a high number of contaminated carcasses after processing which can transmit the pathogen to humans. Since the cecum is the only amplification point of C. jejuni throughout the entire food chain, on-farm intervention measures that reduce colonization of the ceca may have a significant impact on the number of campylobacteriosis cases in humans. However, at present, no effective strategy exists to clear C. jejuni from broiler flocks. As a consequence, the incidence of campylobacteriosis in humans remains strikingly high.

The general scientific aim of this PhD thesis was to develop an intervention measure that consistently reduces C. jejuni numbers in the ceca of broiler chickens by a 1000-fold, which would lead to a reduction in the number of human campylobacteriosis cases by 90%.

Fatty acids are known to possess marked antibacterial activity against a wide range of microorganisms. Therefore, in the first experimental study (chapter 3.1) the potential contribution of medium-chain fatty acids (MCFA) to control C. jejuni in poultry was evaluated. In vitro experiments revealed relatively low minimal inhibitory concentrations (MIC) at pH 6 (of 0.25 to 0.5 mM) for caproic, caprylic and capric acid against C. jejuni compared to other bacterial pathogens (including Salmonella enterica serovar Typhimurium). Also, these MIC values were about 10-fold lower compared to those for short-chain fatty acids against C. jejuni. Time-kill curve experiments showed marked bactericidal properties for capric acid, especially. Therapeutic administration of free caproic, caprylic and capric acid or micro-encapsulated caproic and caprylic acid in the feed at 1% (wt/wt) for the last three days of a 28-day in vivo experiment did not reduce bacterial counts of broilers experimentally inoculated with C. jejuni after two weeks. The same was observed for a water application of MCFA, where an emulsified commercial mixture of caproic, caprylic, capric and lauric acid was added to the drinking water at 0.4% (vol/vol). To examine the protective effect of this emulsion, it was added to the drinking water at the same dose from day-of-hatch onward. Five
days after *C. jejuni* challenge of only a few two-week-old birds (seeders), which act as a transmitting source for the remainder of the animals (contact animals), cecal colonization of both seeders and contact animals, and thus transmission, was not reduced. The inability of MCFA to kill *C. jejuni* in the chicken cecum was validated using a cecal loop experiment, where capric acid was directly injected into the ceca of the animals at concentrations of multiple times the MIC, indicating that *C. jejuni* is protected against the antibacterial activity of MCFA in the broiler cecum. *In vitro* tests in the presence of chicken intestinal mucus indicated that the mucus layer plays a crucial role in this protective effect. Therefore, it was concluded that MCFA are not able to exert their anti-*C. jejuni* properties in the chicken gut at relevant doses and thus will not contribute to *C. jejuni* control once the bacterium reached the ceca. In a final *in vivo* trial of the first study, we investigated the effect of MCFA on the *C. jejuni* colonization threshold in chicks. For this purpose, birds were housed individually to eliminate *C. jejuni* transmission between animals of the same group. We demonstrated that adding the abovementioned emulsion of MCFA to the drinking water of individually housed broilers at 0.4% (vol/vol) resulted in less chicks being colonized 24 hours after inoculation with low (3 × 10^{2} colony-forming units (cfu)) to moderate (2 × 10^{3} cfu) *C. jejuni* numbers. In addition, no viable *C. jejuni* bacteria could be recovered from the treated drinking water.

In conclusion, intestinal mucus is at least in part responsible for forming a protective cecal niche, rendering *C. jejuni* unresponsive to the antibacterial effects of MCFA observed *in vitro*. MCFA may, however, be capable to reduce the probability of *C. jejuni* entrance into a flock if exposure is limited, probably by exerting an antibacterial effect in more proximal sites of the gastro-intestinal (GI) tract.

Several plant-derived compounds are known to possess marked antibacterial activity against a wide range of micro-organisms, including *C. jejuni*. Therefore, in a second study (chapter 3.2) a selection of plant-derived antimicrobial compounds (caffeic, gallic, protocatechuic and vanillic acid, epigallocatechin gallate, trans-cinnamaldehyde and thymol) were screened for their inhibitory and bactericidal properties against *C. jejuni*. The cinnamon-oil ingredient trans-cinnamaldehyde (CIN) and the green tea polyphenol epigallocatechin gallate (EGCG) had the lowest MIC (0.125 mM) of the compounds tested. In addition, CIN was already bactericidal at 0.125 mM while EGCG was not. For this reason, CIN was tested in a broiler chick seeder model in which it was added to the feed in coated form, to enhance survival along the GI tract, at an effective concentration of 0.3% from day-of-hatch for the entire 22-day duration of the experiment. Eight days after inoculation of the seeder animals with *C. je
jejuni, cecal bacterial counts were not reduced. This observation was confirmed in a cecal loop experiment, where CIN concentrations, much higher than the MIC, did not reduce cecal C. jejuni numbers in treated ceca at two and 24 hours after injection. In conclusion, this study shows a marked discrepancy between in vitro and in vivo activity of trans-cinnamaldehyde against C. jejuni.

Given the rather disappointing results obtained when using fatty acids or plant-derived antimicrobial compounds, in a third study (chapter 3.3), a completely different approach was chosen: immune-based intervention measures. Studying passive immunization was chosen over direct vaccination, for two reasons: (1) the interaction of C. jejuni in chicks is of commensal nature and an inefficient immune response is induced during colonization; (2) maternally-derived antibodies are believed to play a key role in protecting broilers against C. jejuni colonization during the first two weeks. This indicates that passive administration of such antibodies may be a promising approach in extending the C. jejuni-free status of the flock up to slaughter.

For this purpose, Campylobacter-free laying hens were repeatedly immunized with either a whole cell protein lysate or only the hydrophobic protein fraction of C. jejuni, using Freund’s Complete (first immunization) and Incomplete (booster immunizations) Adjuvant, after which their eggs were collected. Preventive administration of the hyperimmune yolks to the feed of broiler chicks from day seven onward dramatically reduced cecal colonization of seeders, experimentally inoculated with the homologous C. jejuni strain at 11 days of age. In addition, transmission to contact animals was significantly reduced (hydrophobic protein fraction) or even completely prevented (whole cell protein lysate). In vitro tests using purified immunoglobulin Y (IgY) indicated that C. jejuni motility was unaffected but adhesion to chicken intestinal mucus was enhanced, suggesting that IgY promotes mucosal clearance in vivo. Western Blot analysis and mass spectrometric analysis identified several of the immunodominant antigens of C. jejuni, which may be promising targets for the development of subunit vaccines for C. jejuni control in poultry.

To conclude, where the use of fatty acids or plant-derived antimicrobial products will probably not lead to the ultimate goal of reducing the cecal C. jejuni load of broiler chicks by a 1000-fold, passive immunization seems a very promising alternative intervention measure to reduce C. jejuni colonization in broiler chicks.
SAMENVATTING
SAMENVATTING

*Campylobacter jejuni* is vandaag de belangrijkste oorzaak van gastro-intestinale ziekte veroorzaakt door voedselpathogenen bij de mens wereldwijd en vormt een ernstig gevaar voor de volksgezondheid. Kippen worden aanzien als de belangrijkste bron voor campylobacteriose in de mens. *C. jejuni* is aangepast aan de omgeving van de kippendarm en gekolonizeerde vogels kunnen zeer hoge bacterie aantallen herbergen, voornamelijk in de ceca, zonder echter klinische symptomen te vertonen. Vanaf een leeftijd van twee tot drie weken zijn kippen zeer gevoelig voor *C. jejuni* kolonizatie en van zodra de bacterie in een kippentoom wordt geïntroduceerd, leidt dit tot de kolonizatie van alle dieren in de toom op slachtleeftijd. Dit resulteert in een enorme hoeveelheid gecontamineerde karkassen na behandeling in het slachthuis. *C. jejuni*-besmette karkassen kunnen de pathogeen overdragen naar de mens. Aangezien het cecum het enige punt is waar *C. jejuni* vermeerdering mogelijk is doorheen de volledige voedselketen, zouden maatregelen tijdens de primaire productie gericht op het reduceren van de cecale kolonizatie een grote impact kunnen hebben op het aantal campylobacteriosis gevallen bij de mens. Vandaag is er echter geen doeltreffende interventie maatregel voorhanden die *C. jejuni* kolonizatie in de kip consistent kan reduceren. Als gevolg, blijft het aantal humane campylobacteriose gevallen dramatisch hoog.

Het wetenschappelijk doel van dit onderzoek was om een interventie maatregel te ontwikkelen die consistent tot een 1000-voudige reductie leidt van levende *C. jejuni* kiemen ter hoogte van de ceca van braadkippen. Dit zou het aantal campylobacteriose gevallen bij de mens tot tien maal kunnen reduceren (Rosenquist *et al*., 2003, Messens *et al*., 2007).

Van vetzuren is het geweten dat zij een sterke antibacteriële werking uitoefenen tegen een brede waaier aan micro-organismen. Om die reden werd hun mogelijke bijdrage tot de bestrijding van *C. jejuni* kolonizatie bij pluimvee nagegaan. Hiertoe werd in een eerste studie (hoofdstuk 3.1) de anti-*C. jejuni* eigenschappen van middellange keten vetzuren (MKVZ) nagegaan *in vitro* en werd via verschillende *in vivo* modellen de mogelijkheid van deze zuren om *Campylobacter* kolonizatie in de vleeskip te reduceren onderzocht. Toedienen van deze zuren in hun vrije vorm of ingebed in een matrix, die vrijstelling toelaat ter hoogte van de darm, aan reeds gekolonizeerde kippen leidde niet tot een reductie in cecale *C. jejuni* aantallen. Om het preventieve effect na te gaan werden MKVZ toegediend aan ééndagskuikens tot aan het einde van het experiment. Na twee weken werden...
enkele kuikens (seeders) oraal geïnoculeerd met *C. jejuni*. Deze dieren fungeren als transmissiebron voor de rest van de dieren. Vijf dagen na inoculatie, waren de cecale *C. jejuni* aantallen en transmissie naar contact dieren niet gereduceerd ten opzichte van de controle groep.

Beide waarnemingen werden gevalideerd in een cecaal loop model, waar caprinezuur geïnjecteerd werd in de ceca van de dieren aan concentraties ver boven de minimaal inhibitorische concentratie. Dit doet vermoeden dat *C. jejuni* beschermd is tegen de antibacteriële werking van MKVZ in het cecum van de vleeskip. Via *in vitro* testen werd aangetoond dat intestinale mucus van de kip een belangrijke rol speelt in dit beschermend effect. Er werd besloten dat MKVZ niet in staat zijn om hun anti-*C. jejuni* activiteit uit te voeren in het kippen cecum aan relevante concentraties. Als gevolg zal hun bijdrage tot het bestrijden van *C. jejuni* kolonizatie bij pluimvee beperkt zijn eens de bacteriën de ceca hebben bereikt.

Om na te gaan of MKVZ een anti- *C. jejuni* effect veroorzaken in meer proximale delen van het gastro-intestinaal (GI) stelsel (i.e. in de krop en maag), werden vleeskippen individueel gehuisvest om op die manier transmissie van gekolonizeerde naar niet-gekolonizeerde dieren uit te sluiten. Daags na experimentele inoculatie werden de ceca gecontroleerd op aanwezigheid van *C. jejuni*. Preventieve administratie van MKVZ resulteerde in een hogere kolonizatie drempelwaarde. Deze observatie kon echter niet worden gevalideerd in een seeder model, wat de situatie in de kwekerij beter benadert. Er kan daarom besloten worden dat de potentiële bijdrage van MKVZ tot het reduceren van *C. jejuni* kolonizatie in pluimvee eerder klein is.

Ook van verschillende plantaardige componenten is het geweten dat ze een uitgesproken antibacteriële werking kunnen vertonen. Daarom werd in een tweede luik (hoofdstuk 3.2) een selectie van plantaardige antibacteriële componenten gescreened op groei-inhiberende en bactericide eigenschappen tegenover de gebruikte *C. jejuni* stam. Uit de resultaten van de *in vitro* testen bleek *trans*-cinnamaldehyde de meest veelbelovende van de geteste component te zijn. Echter, verschillende *in vivo* experimenten, gebruik makend van een seeder, een cecaal loop en een therapeutisch model faalden om enige reductie in cecale *C. jejuni* aantallen met zich mee te brengen, zelfs wanneer *trans*-cinnamaldehyde werd ingekapseld om vroegtijdige afbraak doorheen het GI stelsel te minimaliseren.
Gezien de niet echt bemoedigende resultaten die werden bekomen met vetzuren en plantaardige antibacteriële componenten werd in een derde en laatste studie (hoofdstuk 3.3) voor een andere aanpak geopteerd: controle maatregelen gebaseerd op immuuniteit. Er werd beslist om in plaats van directe vaccinatie het effect van passieve immunisatie op bestrijding van *C. jejuni* bij pluimvee na te gaan, en dit om twee redenen: (1) de interactie van *C. jejuni* en de kip is commensaal van aard en daarom wordt slechts een inefficiënte immuun respons opgewekt tijdens kolonizatie; en (2) maternale antilichamen worden verondersteld een zeer belangrijke rol te spelen in de bescherming van vleeskippen tegen *C. jejuni* kolonizatie tijdens de eerste twee weken na uitkippen. Dit doet vermoeden dat passieve toediening van dergelijke antilichamen een veelbelovende aanpak kan zijn om de *C. jejuni*-vrije status van braadkippen te verlengen tot slachtleef tijd.

Hiertoe werden *Campylobacter*-vrije legkippen herhaaldelijk geïmmunizeerd met een volledig cellysaat of enkel de hydrofobe eiwitfractie van *C. jejuni* waarna hun eieren werden verzameld. Preventieve toediening van de immunoglobuline Y (IgY)-rijke dooiers aan het voeder van vleeskippen leidde tot een massale reductie in cecale *C. jejuni* aantallen in seeders en leidde tot een drastische reductie (hydrofobe eiwitfractie) of zelfs volledige eliminatie (volledig cellysaat) van transmissie naar contactdieren na experimentele inoculatie met de homologe *C. jejuni* stam. *In vitro* testen met opgezuiverd IgY toonden aan dat *C. jejuni* motilititeit niet werd aangetast maar dat adhesie aan intestinale mucus van de kip gepromoot werd, wat een verhoogde mucosale clearance doet vermoeden *in vivo*. Via Western Blot analyse en massa spectrometrie werden verschillende van de immunodominante antigenen van *C. jejuni* in de kip geïdentificeerd, die mogelijks kunnen worden gebruikt voor de ontwikkeling van subunit vaccins ter bestrijding van *C. jejuni* kolonizatie in pluimvee.

Dus, daar waar het gebruik van vetzuren en plantaardige antimicrobiële componenten hoogst waarschijnlijk niet zal leiden tot een 1000-voudige reductie in het aantal cecale *C. jejuni* bacteriën in vleeskippen, lijkt het gebruik van passieve immunisatie een veelbelovende alternatieve maatregel in het bestrijden van *C. jejuni* kolonizatie bij pluimvee.
Personalia


Vanaf oktober 2008 trad hij in dienst als doctoraatsbursaal aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten, waar hij gedurende vier jaar onderzoek verrichtte naar Campylobacter controle bij pluimvee.

David Hermans is auteur van meerdere publicaties in nationale en internationale tijdschriften en hij presenteerde zijn onderzoeksresultaten op een internationaal congres aan de hand van een poster en mondelinge presentatie.
Publications


Proceedings & abstracts


Oral presentation

DANKWOORD
DANKWOORD

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