Evaluation of passive and active immunisation strategies to control *Campylobacter* infections in broiler chickens

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°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micromolar
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CC	Clonal complex
C. coli	*Campylobacter coli*
cfu	Colony forming units
C. hepaticus	*Campylobacter hepaticus*
CjaA	Cja subunit A
CjaD	Cja subunit D
C. jejuni	*Campylobacter jejuni*
CSB	Colombia Sheep Blood agar
DNA	Deoxyribonucleic acid
EC	Ethical Committee
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium*
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay

EU	European Union
EYP	Egg yolk powder
FCA	Freund's complete adjuvants
FIA	Freund's incomplete adjuvants
FlaA	Flagellin subunit A
g	Gram
GEM	Gram-positive enhancer matrix
h	Hour
HBSS	Hank's Balanced Salt Solution
HEYP	Hyperimmune egg yolk powder
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
ILVO	Flanders Research Institute for Agriculture, Fisheries and Food
kg	Kilogram
L	Litre
L. acidophilus	*Lactobacillus acidophilus*
LB	Luria Broth

LBC	Lohmann Brown-Classic layer hens
L. brevis	*Lactobacillus brevis*
L. crispatus	*Lactobacillus crispatus*
L. fermentum	*Lactobacillus fermentum*
LLC	Lohmann LSL-Classic layer hens
log	Logaritm
L. salivarius	*Lactobacillus salivarius*
m	Meter
MAB	Maternal antibodies
Maru HB380	Maru Hatcher & Brooder 380
mCCDA	Modified charcoal cefoperazone deoxycholate agar
mg	Milligram
min	Minute
mL	Millilitre
MLST	Multilocus sequence typing
mM	Millimolar
MOMPs	Major outer membrane proteins
n	Number
NB2	Nutrient Broth No. 2
NEYP	Non-immunized egg yolk powder
OD	Optical density
OMPs	Outer membrane proteins

OMVs	Outer membrane vesicles
p	P-value
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
p_{eq}	Equivalent p-value
pfu	Plaque forming units
qPCR	Quantitative PCR
rCjaAD	Recombinant CjaA expressing CjaD epitopes
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TMB	3,3’,5,5’-tetramethyl benzidine
vol	Volume
wt	Weight

CHAPTER 1: INTRODUCTION

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Campylobacteriosis has been the most important bacterial zoonosis since 2005 and 246,158 cases were reported in the EU in 2017 (EFSA, 2018). However, the disease is highly underreported and it is estimated that there are close to 9 million cases annually, resulting in an estimated annual cost of €2.4 billion (EFSA, 2018). Poultry products, especially from chickens, form the main source for human infection (Berndtson et al., 1992; Friedman et al., 2004; EFSA, 2018), therefore measures to control this bacterium in poultry should be developed to limit this zoonosis.

This thesis gives an overview of the current situation in humans and poultry, including present and potential measures to control the bacterium in chickens. The high number of human campylobacteriosis cases indicates that the current implementation is not sufficient. Since immunisation could be an effective method to limit *Campylobacter* in poultry (de Zoete et al., 2007), the experimental chapters of this thesis are focused on passive and active immunisation, followed by a discussion on implementing these and other measures in the poultry industry.

1. *Campylobacter* spp. and human campylobacteriosis

1.1. CAMPYLOBACTER SPP.

Campylobacter was first described by Theodor Escherich in 1886 and was considered a *Vibrio*-like species until the introduction of the genus *Campylobacter* by Sebald and Véron in 1963 (Debruyne et al., 2008). The taxonomy of the genus is given in Table 1. The name '*Campylobacter'* is derived from the Greek words *campylos* (curved) and *baktron* (rod). These bacteria are indeed curved, S-shaped or spiral rods, 0.2-0.9 µm wide and 0.5-5 µm long. They are Gram-negative and non-sporulating, do not ferment or oxidize carbohydrates and therefore obtain their energy from amino acids and Krebs cycle intermediates (Westfall et al., 1986). Motility is obtained by a polar flagellum at one or both sides of the cell. *Campylobacter* only grows at low oxygen levels and a minimum temperature of 30°C, optimally at 5% O_2, 10% CO_2 and 42°C (Park, 2002). These bacteria only multiply in some warm-blooded animals, but despite these growth requirements, they are widely prevalent in the environment (Snelling et al., 2005). Since *Campylobacter* is a highly diverse genus, a result of the presence of highly mutable sites (Jerome et al., 2011) and interspecies recombination (Boer et al., 2002), different strains typing methods are available. Currently, multilocus sequence typing (MLST) is the gold standard (Duarte et al., 2016). Based on the sequence data of seven housekeeping genes, the strains are classified into clonal complexes (CCs) and sequence types (STs).

Table 1. Taxonomy of the genus *Campylobacter*.

Taxonomic rank	Name
Domain	Bacteria
Phylum	Proteobacteria
Class	Epsilonproteobacteria
Order	Campylobacterales
Family	Campylobacteraceae
Genus	*Campylobacter*

1.2. HUMAN CAMPYLOBACTERIOSIS

Campylobacter has been the most commonly reported bacterial gastrointestinal pathogen in humans in the European Union (EU) since 2005 (EFSA, 2018). In 2017, a total of 246,158 confirmed cases of human campylobacteriosis, or 64.8 per 100,000 inhabitants, were reported. Most of these cases are sporadic, however outbreaks occur (Snelling et al., 2005). Many cases remain unreported and therefore the true incidence of campylobacteriosis is not known (Havelaar et al., 2013). It is estimated that that there are close to 9 million cases annually, resulting in an estimated annual cost of €2.4 billion (EFSA, 2018). Campylobacteriosis is mainly caused by *Campylobacter jejuni* (*C. jejuni*) and *C. coli*, accounting for respectively 84.4% and 9.2% of the cases in 2017 (EFSA, 2018). For simplicity, these species are hereafter referred to as *Campylobacter*. After ingestion and successful passage of the stomach acid barrier, *Campylobacter* colonizes the distal ileum and colon (Blaser and Engberg, 2008). There, the bacterium colonizes the mucus layer and adheres to epithelial surfaces. It damages the epithelial cellular function, impeding the absorptive capacity of the intestine and leading to diarrhea (Blaser and Engberg, 2008). Other symptoms include fever, abdominal cramps and sometimes vomiting and bloody diarrhea (Gillespie et al., 2006). In rare cases, complications

may occur, which may ultimately lead to inflammatory bowel disease, reactive arthritis or Guillain-Barré Syndrome (Butzler, 2004; Snelling et al., 2005).

The infective dose can be as low as 500 bacteria (Snelling et al., 2005), depending on the strain virulence and the host susceptibility (Johnson et al., 1984; Blaser et al., 1986; Black et al., 1988). The incubation period varies from 2 to 5 days (Istre et al., 1984; Korlath et al., 1985; Blaser, 1987) and symptoms typically last for 1 week (Sjögren et al., 1989; Endtz et al., 1993). These symptoms are hard to distinguish from salmonellosis and shigellosis and definitive diagnosis is only possible by detection of *Campylobacter* in the stool of the patient (Blaser and Engberg, 2008). Since the disease is mostly self-limiting, no antimicrobial therapy is needed and replacement of fluids and electrolytes lost through diarrhea and vomiting should be sufficient (Butzler, 2004). However, if the symptoms continue, if complications occur and for patients at a higher risk, such as the elderly, the use of antimicrobials might be recommended (Reed et al., 1996; Manfredi et al., 1999; Miller et al., 2005).

Campylobacter infection mainly originates from contaminated meat (Deming et al., 1987; Eberhart-Phillips et al., 1997; Studahl and Andersson, 2000; Effler et al., 2001; Wingstrand et al., 2006), but can also occur via contaminated water (Young et al., 2007), untreated milk (Doyle and Roman, 1982), direct contact with animals, such as cattle and pets (Tenkate and Stafford, 2001; de Haan et al., 2010) and raw vegetables, contaminated at the farm or during food handling (Verhoeff-Bakkenes et al., 2011; Gardner et al., 2011). In general, poultry meat is considered the main source of human *Campylobacter* infection (Stern and Kazmi, 1989; Berndtson et al., 1992; Nielsen et al., 1997; Vellinga and Van Loock, 2002; Nadeau et al., 2002; Friedman et al., 2004; Zorman et al., 2006; Colles et al., 2008; Wilson et al., 2008; Gonzalez et al., 2009; Lindmark et al., 2009; Wassenaar et al., 2009; Müllner et al., 2010). Human infection occurs by improper food handling, such as cross-contamination, and subsequent consumption of contaminated food (Berndtson et al., 1992; Friedman et al., 2004).

2. *Campylobacter* spp. in poultry

2.1. PATHOGENESIS

In contrast to human infection, *Campylobacter* colonization in chickens generally does not cause clinical symptoms (Beery et al., 1988; Evans and Sayers, 2000; Keener et al., 2004; Meade et al., 2009b). Chickens can become colonized after ingestion of as low as 35 colony forming units (cfu) *C. jejuni* (Stern et al., 1988), however the colonization capacity of the bacterium depends on the strain (Stern et al., 1988; Ringoir and Korolik, 2003; Hänel et al., 2009), its origin (Korolik et al., 1998), inoculation dose and the chicken breed (Stern et al., 1990a; Boyd et al., 2005) and age (Sahin et al., 2001, 2003b). An increased colonization capacity and virulence after *in vivo* passage has been observed (Stern et al., 1988; Sang et al., 1989; Cawthraw et al., 1996; Hänninen et al., 1999; Boer et al., 2002; Ringoir and Korolik, 2003), however, there is some controversy about this since others did not find changes after passage (Nielsen et al., 2001; Manning et al., 2001; Konkel et al., 2007). When *Campylobacter* reaches the cecum, it mainly colonizes the mucus layer and intestinal crypts, where it starts multiplying (Meade et al., 2009b; Hermans et al., 2012b). As a result, chickens can carry large numbers of *Campylobacter* within 24 hours after ingestion, generally 10^6 to 10^8 cfu/g cecal content (Beery et al., 1988; Coward et al., 2008; Meade et al., 2009b).

This colonization is the result of three key events: chemotaxis, epithelial cell adhesion and invasion. *Campylobacter* is attracted to mucins and glycoproteins and migrates through flagellar motility (Vegge et al., 2009; Lertsethtakarn et al., 2011; Facciolà et al., 2017). After mucus penetration, the bacteria adhere to epithelial cells (Guerry, 2007) and some invade these cells (Van Deun et al., 2008b). Since *Campylobacter* cannot multiply intracellularly, the bacteria evade, multiply in the mucus and re-invade to escape mucosal clearance (Van Deun et al., 2008b). Next to these main events, also additional mechanisms play a role, such as

several stress responses, multidrug and bile resistance regulation, iron regulation and energy metabolism (Hermans et al., 2011a).

Often, naturally infected chickens are colonized by more than one sero- or genotype at the same time (van de Giessen et al., 1992; Jacobs-Reitsma et al., 1995; Konkel et al., 2007). Various strains may succeed each other, this is mainly determined by the chicken host and not by host microbiota (Skånseng et al., 2007; Ridley et al., 2008b). To date, little is known about the interaction of *Campylobacter* with the host microbiome, which may act as a colonization barrier and influence to some extent whether *Campylobacter* will be able to colonize the chicken gut (Keeney and Finlay, 2011).

Young chicks develop innate immunity a few days post-hatch (Friedman et al., 2003; Bar-Shira and Friedman, 2006). In chicken embryo's, an increased expression of avian β-defensins has already been detected (Meade et al., 2009a). Adaptive immunity is developed three weeks after hatch (Friedman et al., 2003; Bar-Shira and Friedman, 2006). After *C. jejuni* colonization, both an innate and adaptive immune response are detected (Cawthraw et al., 1994; Widders et al., 1998). Some studies demonstrated a mild inflammatory response and infiltration of proinflammatory cells in mucosal tissues, however no pathological signs were seen (Smith et al., 2008; Larson et al., 2008). The adaptive response consists of serum IgM, IgY and IgA and mucosal secretory IgA, the latter preventing sub-epithelial translocation or returning translocated bacteria without inflammatory response (Brisbin et al., 2008). Despite the initiation of this immune response, *C. jejuni* is able to evade this response and the bacteria are not cleared from the chicken gut but establish persistent colonization.

Anti-*Campylobacter* antibodies are highly prevalent in breeder flocks, egg yolks and young broiler chicks (Sahin et al., 2001). Maternal antibodies, mainly IgY (Hermans et al., 2014), produced by hens are transferred via the egg yolk to the offspring. These antibodies probably

protect the chicks from *Campylobacter* colonization during early life since most flocks only become colonized at an age of two to three weeks (Jacobs-Reitsma et al., 1995; Evans and Sayers, 2000; Sahin et al., 2001, 2003b; Herman et al., 2003; van Gerwe et al., 2009; Cawthraw and Newell, 2010).

The fact that chickens elicit an immune response when colonized by *Campylobacter*, puts its commensal nature under discussion (Humphrey et al., 2014). Moreover, *Campylobacter* has been isolated from the thymus, spleen, liver, gallbladder, bursa of Fabricius, reproductive tract and ovarian follicles of infected chickens (Camarda et al., 2000; Buhr et al., 2002; Hiett et al., 2003; Cox et al., 2005, 2009; Young et al., 2007; Van Deun et al., 2008b; Meade et al., 2009b; Hermans et al., 2012b; Pielsticker et al., 2016; Facciolà et al., 2017) and *Campylobacter*-induced diarrhea, growth retardation and jejunal villus atrophy have been reported (Sanyal et al., 1984; Sang et al., 1989; Lam et al., 1992; Lamb-Rosteski et al., 2008). Recently, a novel *Campylobacter* species, *C. hepaticus*, has been described, which was isolated from chickens with spotty liver disease (Van et al., 2016). In general, however, chickens do not seem to suffer from *Campylobacter* colonization (Beery et al., 1988; Evans and Sayers, 2000; Keener et al., 2004; Meade et al., 2009b).

2.2. EPIDEMIOLOGY

2.2.1. ENVIRONMENTAL SOURCES

Campylobacter is highly prevalent in the environment (Newell, 2002; Murphy et al., 2006), especially in surface water (Bull et al., 2006; Messens et al., 2009). It is still unclear how such a fragile bacterium is able to survive stresses outside the natural host (Murphy et al., 2006). One explanation could be that the *Campylobacter* genome contains highly mutable sites, facilitating rapid adaptation in a novel environment (Jerome et al., 2011).

The bacteria are also present in the natural intestinal microbiota of various animals, such as broilers, layer hens, turkeys, cattle, pigs, sheep, dogs and ostriches (van de Giessen et al., 1996; Nielsen et al., 1997; On et al., 1998; Newell, 2002; Siemer et al., 2004; Whyte et al., 2004; Abulreesh et al., 2006; Young et al., 2007; Ridley et al., 2008a, 2011; Zweifel et al., 2008; Gilpin et al., 2008; Ragimbeau et al., 2008; Ellis-Iversen et al., 2009; Huang et al., 2009; Hakkinen et al., 2009; Ogden et al., 2009; Jokinen et al., 2011; Allen et al., 2011). A correlation has been found between *C. jejuni* genotypes from broiler flocks and other animals, such as cattle, pigs and laying hens, present at the same farm (van de Giessen et al., 1996; Ridley et al., 2008a; Zweifel et al., 2008; Ellis-Iversen et al., 2009; Allen et al., 2011). In general, *C. jejuni* is the most isolated species (McDowell et al., 2008; Zweifel et al., 2008; Kuana et al., 2008; Ellis-Iversen et al., 2009; Nather et al., 2009; Messens et al., 2009; EFSA, 2011; Jorgensen et al., 2011), however *C. coli* is most prevalent in pigs (EFSA, 2011) and has been isolated most often in Spain, Luxembourg, Slovenia, and Bosnia and Herzegovina (Zorman et al., 2006; EFSA, 2011).

2.2.2. RISK FACTORS FOR COLONIZATION

Initial colonization of broiler flocks with *Campylobacter* depends on many factors, such as:

- prevalence in the environment (Stas et al., 1999; Petersen and Wedderkopp, 2001; Wedderkopp et al., 2003; Kudirkienė et al., 2010),
- colonization capacity of the strain (Stas et al., 1999; Petersen and Wedderkopp, 2001; Wedderkopp et al., 2003; Kudirkienė et al., 2010),
- infective dose (Stas et al., 1999; Petersen and Wedderkopp, 2001; Wedderkopp et al., 2003; Kudirkienė et al., 2010),
- age of the animals (Barrios et al., 2006),

- flock size (Berndtson et al., 1996b; Barrios et al., 2006),
- climate, seasons and weather conditions, such as a higher prevalence during summer months and after rainfall (McDowell et al., 2008; Ellis-Iversen et al., 2009; Ellerbroek et al., 2010; Jorgensen et al., 2011),
- ineffective hygiene measures (McDowell et al., 2008; Allen et al., 2011),
- the presence of other colonized animals (van de Giessen et al., 1996; Zweifel et al., 2008; Ellis-Iversen et al., 2009),
- the presence of rodents, flies and their larvae (Berndtson et al., 1996a; Hald et al., 2004, 2008; Nichols, 2005; Hazeleger et al., 2008),
- contaminated surface water (Messens et al., 2009),
- contaminated personnel and farm equipment, such as trucks, forklifts, pallets, crates and footwear (Ramabu et al., 2004) and
- partial depopulation (Allen et al., 2008; Patriarchi et al., 2011), although this is under discussion (Russa et al., 2005; Barrios et al., 2006; Nather et al., 2009).

The fact that there are many different risk factors complicates source attribution of *Campylobacter* contamination. Moreover, these factors are not completely independent of each other (Jorgensen et al., 2011). For example, during the warm summer months, increased numbers of rodents and flies can be found on the farm, and rainfall creates surface water reservoirs. Therefore, all of these factors should be considered when introducing measures to control *Campylobacter*.

2.2.3. FLOCK COLONIZATION

Worldwide, on average 60 to 80% of broiler flocks are contaminated with *Campylobacter* at slaughter age (Herman et al., 2003; Rasschaert et al., 2006; Reich et al., 2008; Kuana et al.,

2008). The chicks are initially colonized by horizontal transmission from environmental sources (van de Giessen et al., 1992; Jacobs-Reitsma et al., 1995; Bull et al., 2006; Patriarchi et al., 2011). Vertical transmission from breeder hens or transmission from a previous flock in the same housing are unlikely (van de Giessen et al., 1992; Jacobs-Reitsma et al., 1995; Sahin et al., 2003a; Bull et al., 2006; Callicott et al., 2006; Patriarchi et al., 2011; Silva et al., 2011), in contrast to *Salmonella* infection (Carrique-Mas et al., 2008). However, subsequent flocks may be infected with the same strain if the strain persists in the environment (Petersen and Wedderkopp, 2001; Wedderkopp et al., 2003; Ellerbroek et al., 2010).

Once a broiler in the flock is infected, *Campylobacter* easily spreads to the other chickens via the fecal-oral route, which can be explained by high shedding via the faeces, contamination of drinking water, floor shavings and feed and coprophagous behaviour of the chicks (Evans, 1992; Gregory et al., 1997; Newell and Fearnley, 2003; Herman et al., 2003; van Gerwe et al., 2009; Messens et al., 2009; Sparks, 2009). The majority of chickens are colonized a few days later (van Gerwe et al., 2009), or a week after initial colonization of one single seeder bird (Stern et al., 2001b). van Gerwe et al. (2009) also calculated a transmission rate of 2.37 new cases per colonized chick per day, infecting 95% of all broilers one week after initial colonization for a flock of 20,000 animals. Once infected, flocks generally remain colonized until slaughter (Beery et al., 1988; Jacobs-Reitsma et al., 1995; Evans and Sayers, 2000; Stern et al., 2001b; van Gerwe et al., 2009).

2.2.4. TRANSPORTATION AND CARCASS CONTAMINATION

Stress during transportation to the slaughterhouse was shown to increase *Campylobacter* numbers in broiler ceca (Stern et al., 1995) and faeces (Whyte et al., 2001). Since transport crate disinfection is often ineffective and these crates can be contaminated when re-used

(Ridley et al., 2011), transportation might be involved in *Campylobacter* contamination of negative flocks (Hastings et al., 2011; Patriarchi et al., 2011).

Campylobacter contamination was found to be variable both within and between batches in Belgian slaughterhouses (Seliwiorstow et al., 2015). A significant correlation can be found between the *Campylobacter* colonization rate of broiler chickens during rearing and bacterial numbers on their carcasses after processing, indicating that the live flock itself is an important source for carcass contamination (Rosenquist et al., 2003, 2006; Herman et al., 2003; Rasschaert et al., 2006; Reich et al., 2008; Colles et al., 2010). During defeathering and especially evisceration, carcasses can be contaminated by visceral rupture of the ceca and leaking of contaminated faeces from the cloaca (Berrang et al., 2001; Smith et al., 2007; Allen et al., 2008; Boysen and Rosenquist, 2009; Seliwiorstow, 2015). However, other sources, such as cross-contamination within and between flocks, also play a role (Rosenquist et al., 2003; Herman et al., 2003; Rasschaert et al., 2006; Allen et al., 2008; Normand et al., 2008) and it is shown that the diversity of isolated *Campylobacter* genotypes can be increased during the slaughter process (Colles et al., 2010). Indeed, carcasses from *Campylobacter*-negative batches were found to become contaminated when processed immediately after *Campylobacter*-positive batches and the numbers on the previously negative carcasses were influenced by the colonization level of those positive broilers (Seliwiorstow, 2015). This can be explained by contamination of the slaughter environment by colonized birds and persistence of *Campylobacter*, even after cleaning and decontamination (Newell et al., 2001; Miwa et al., 2003; Peyrat et al., 2008; Ellerbroek et al., 2010). Furthermore, Seliwiorstow (2015) identified risk factors associated with increased *Campylobacter* numbers on carcasses, such as incorrect setting of plucking, evisceration and cloaca cutter machines, and too low a scalding temperature. Interestingly, because all these factors were existing variations of routine practices, they can easily be optimized.

As a consequence of the cross-contamination in the slaughterhouse, on average 60% to 80% of poultry carcasses are reported to be contaminated with *Campylobacter* worldwide (Suzuki and Yamamoto, 2009; EFSA, 2010; Müllner et al., 2010), mostly *C. jejuni* (Rasschaert et al., 2006; Kuana et al., 2008; Suzuki and Yamamoto, 2009; EFSA, 2010). In the EU, 37.4% of fresh broiler meat was found to be contaminated with *Campylobacter* sp. in 2017 (EFSA, 2018). Highly contaminated products pose the main risk for consumer health (Nauta et al., 2009), therefore aiming at reduced *Campylobacter* numbers on carcasses may be sufficient to control campylobacteriosis. Nearly all parts of contaminated carcasses can be a source for human disease, both fresh, chilled and frozen (Berndtson et al., 1992).

3. *Campylobacter* control in poultry

3.1. RATIONALE FOR INTERVENING AT THE PRIMARY CHICKEN PRODUCTION

Contaminated meat constitutes the link between infected poultry and human disease and limiting *Campylobacter* contamination on poultry carcasses would reduce human campylobacteriosis cases (Hermans et al., 2011b). In the EU, up to 40% of human campylobacteriosis cases can be attributed to *Campylobacter*-contaminated chicken meat and up to 80% of the cases to the chicken reservoir as a whole, when including pathways other than food (EFSA, 2010). Chickens can carry up to 10 $\log_{10}$ cfu of *Campylobacter* spp. in their cecal content (USDA, 2019) and a pre-harvest criterion of 7 $\log_{10}$ cfu/g of *Campylobacter* spp. in pooled caecal samples was recommended by the Food Safety Authority of Ireland (FSAI, 2011). Several quantitative risk analyses have been developed estimating the potential impact of *Campylobacter* control during poultry production (Nauta et al., 2009). According to one model, reducing *Campylobacter* numbers on broiler carcasses by 2 $\log_{10}$ would result in a 30-fold reduction of human campylobacteriosis cases (Rosenquist et al., 2003). Another risk assessment predicted that a 1, 2 or 3 $\log_{10}$ reduction of *Campylobacter* numbers on carcasses would reduce human campylobacteriosis incidence by respectively 48%, 85% and 96% (Messens et al., 2007). Finally, reducing cecal *Campylobacter* colonization by 3 $\log_{10}$ was estimated to reduce carcass contamination by 2 $\log_{10}$ and human campylobacteriosis incidence by 90% (Rosenquist et al., 2003; Neal-McKinney et al., 2014).

Reduction of carcass and, consequently, meat contamination can be achieved directly by treatment of the carcass surface (Rosenquist et al., 2006; Boysen and Rosenquist, 2009), or indirectly by on-farm practices reducing *Campylobacter* colonization in broilers during rearing (Lin, 2009). Many physical or chemical carcass treatment methods, such as irradiation

and treatment with organic acids, are not authorized in the EU or accepted by the public opinion (MacRitchie et al., 2014; Seliwiorstow, 2015). When aiming at reducing on-farm *Campylobacter* colonization, one should consider the difference between prevention and therapeutic reduction: preventive measures intend to reduce the chance that birds get infected, while therapeutic interventions try to reduce bacterial numbers in already colonized chicks, typically before slaughter.

Several measures can be considered to achieve this reduction, including hygiene and biosecurity (Section 3.2), drinking water treatment (Section 3.3), plant-derived feed additives (Section 3.4), probiotics, prebiotics and competitive exclusion (Section 3.5), bacteriocins (Section 3.6), bacteriophages (Section 3.7) and active and passive immunisation (Section 3.8). Unless indicated otherwise, the results described in these sections were obtained under experimental settings and similar protective effects under commercial conditions cannot be guaranteed. Furthermore, improving bird health and welfare was suggested to possibly reduce *Campylobacter* colonization, since lower welfare levels were found to be associated with higher colonization levels (Bull et al., 2008). Selecting for improved immunity instead of solely focussing on growth characteristics when breeding, may be beneficial for the birds' resistance against diseases (Swaggerty et al., 2009). Antibiotics should not be used because of emerging resistance, complicating human treatment of severe campylobacteriosis cases (Dibner and Richards, 2005; Zhu et al., 2006).

3.2. HYGIENE AND BIOSECURITY

To prevent *Campylobacter* introduction into the stable, enough attention should be paid to hygiene and biosecurity measures (Hermans et al., 2012c). First of all, there is a high risk of contamination by the personnel (Newell et al., 2011). Therefore strict hygiene measures

should be applied, such as the correct use of disinfection footbaths, changing work clothes and footwear between stables and washing hands before and after entering a stable (van de Giessen et al., 1996; Evans and Sayers, 2000; Ridley et al., 2011; Newell et al., 2011; Ghareeb et al., 2013; Meunier et al., 2016a). In between production rounds, care should be taken that the stables and equipment are cleaned and disinfected properly (Ridley et al., 2011; Newell et al., 2011). Since rodents and flying insects, especially flies, are important sources of *Campylobacter* contamination, control programs against these vectors are necessary to limit *Campylobacter* introduction in the stables (Bahrndorff et al., 2013; Allain et al., 2014). In one study, placing fly screens in broiler houses reduced the prevalence of *Campylobacter*-colonized flocks from 51.4% to 15.4% (Hald et al., 2007).

Introduction of hygiene and biosecurity measures has been shown to reduce *Campylobacter* prevalence from 66% and 100% to respectively 22% and 42% in two Dutch farms (van de Giessen et al., 1998), from 80% to 40% in the UK (Gibbens et al., 2001) and from 43% in 2002 to 27% in 2007 in Denmark (Rosenquist et al., 2009). Currently, Belgian researchers are investigating how farms with a high rate of *Campylobacter* contamination can reduce this rate by improving biosecurity measures (ILVO, 2017). Taken together, biosecurity clearly impacts *Campylobacter* colonization in broiler flocks. However, these measures need to be applied properly to be effective and will not be sufficient alone.

3.3. DRINKING WATER TREATMENT

Since drinking water is an important transmission route after initial flock infection, reducing *Campylobacter* numbers in the drinking water could limit its spread through the flock (Hermans et al., 2011b). Water chlorination reduced the risk for *Campylobacter* colonization under experimental settings (Newell and Fearnley, 2003; Ellis-Iversen et al., 2009), but

Campylobacter prevalence was not reduced under commercial conditions (Stern et al., 2002). Organic acids were shown to prevent *Campylobacter* transmission through the flock (Chaveerach et al., 2002, 2004a), probably by reducing bacterial numbers in drinking water and the broiler crop (Hermans et al., 2011b). Also, *Campylobacter* contamination in the crop and pre-chill carcasses was reduced when administering lactic acid in the drinking water before slaughter (Byrd et al., 2001). Therapeutic addition of monocaprin to drinking water reduced *C. jejuni* numbers of infected birds, however transmission was not prevented (Hilmarsson et al., 2006). Addition of allicin in drinking water did not have an effect on cecal *C. jejuni* colonization levels in broilers, despite *in vitro* anti-*C. jejuni* activity (Robyn et al., 2013). Administration of a mixture of the medium chain fatty acids caproic, caprylic, capric and lauric acid to the drinking water of broilers prevented survival of *C. jejuni* in the water, however, colonization and transmission of the bacteria in the broilers was not significantly reduced, indicating that other transmission routes are also important (Hermans et al., 2012a).

3.4. FEED ADDITIVES

Changes in feed composition can influence the gastrointestinal microbial composition and *Campylobacter* numbers in the chicken gut. For example, administering plant-protein-based feed (including soybean meal, canola meal and corn-gluten meal) to broiler chickens reduced cecal *Campylobacter* colonization compared to birds administered animal-protein-based feed (including meat meal, poultry-byproducts meal, fish meal and feather meal) or a combination of the two (resp. 4.9, 6.3 and 5.8 $\log_{10}$ cfu/g cecum) (Udayamputhoor et al., 2003).

Administration of plant-derived feed additives may contribute to reducing *Campylobacter* numbers. Fatty acids, the most researched feed additives, are generally considered beneficial for gut health, because of their antimicrobial activity against many 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). However, continuous addition of 0.05% butyrate did not reduce cecal *Campylobacter* colonization although *in vitro* experiments seemed promising (Van Deun et al., 2008a). *C. jejuni* colonization in broiler chicks could be prevented when supplementing a combination of 2% formic acid and 0.1% sorbate (Skånseng et al., 2010). Administration of the medium-chain fatty acid caprylic acid yielded variable results. While Solis de los Santos et al. (2008) succeeded in reducing *Campylobacter* colonization in chicks by 2 $\log_{10}$ using a dose of 0.35-1.4%, no such protection was obtained by Hermans et al. (2012a) using a dose of 0.4%. In the latter study, also no inhibition of *Campylobacter* was obtained by using 0.4% caproic and capric acid. Administering a medium-chain fatty acid mixture to broiler feed at a dose of 1% was shown to increase the median infective dose from 2.5 to 4.8 $\log_{10}$ cfu (van Gerwe et al., 2010). A combination of 0.12% monocaprin and 0.02% polysorbate was able to reduce *Campylobacter* numbers in colonized animals by 1-2 $\log_{10}$, but not to prevent transmission to non-infected birds (Hilmarsson et al., 2006).

Also other plant-derived additives were shown to have a bactericidal activity *in vitro*, especially the cinnamon oil trans-cinnamaldehyde (Friedman et al., 2002). However, no preventive or therapeutic effect was obtained *in vivo* using a dose of 0.3% (Hermans et al., 2011c). The same applies to allicin using a dose of 25 mg/kg (Robyn et al., 2013).

In conclusion, although promising results with these additives were obtained *in vitro*, no consistent *in vivo* protection could be obtained. A plausible explanation is premature metabolic breakdown of these compounds in the broiler chicken gastrointestinal tract (Wittschier et al., 2007), which can be bypassed by coating on or encapsulating in a carrier (Van Immerseel et al., 2004b). *Campylobacter* may also be protected by the mucus layer ('protective niche theory'), since direct injection of a concentrated sodium caprate solution

(200µL, 75 mM) in the broiler cecum did not prevent or reduce *Campylobacter* colonization (Hermans et al., 2010). Prophylactic supplementation seems more promising than reducing *Campylobacter* numbers in colonized birds therapeutically by administering a pulse dose before slaughter. However, the applicability in the field is questionable, since the *in vivo* results are generally disappointing.

3.5. PROBIOTICS, PREBIOTICS AND COMPETITIVE EXCLUSION

Modification of the gut microbiota, improving gut health or stimulating microorganisms that compete with *Campylobacter*, may improve the chicken's resistance to *Campylobacter* colonization. For this, generally health-promoting microorganisms, competitive exclusion cultures or compounds stimulating these species can be administered.

Some administered undefined bacterial mixtures were able to control *C. jejuni* colonization in young chicks (Soerjadi et al., 1982; Soerjadi-Liem et al., 1984), however no such protection was found by Stern et al. (1988). The efficacy varied depending on culture preparation methods and storage (Stern, 1994; Stern et al., 2001a; b).

Probiotics, defined beneficial microbiota, were investigated subsequently for their ability to reduce *Campylobacter* colonization. Supplementation of 3.3×10^7 cfu/L *Lactobacillus acidophilus* (*L. acidophilus*) and *Enterococcus faecium* was able to reduce *C. jejuni* fecal shedding and jejunal colonization in broilers, respectively by 70% and 27% (Morishita et al., 1997). Administering 1-100g/kg dried yeast *Saccharomyces boulardii* did not result in a reduction of cecal *Campylobacter* colonization (Line et al., 1998). *L. acidophilus, L. fermentum, L. crispatus* and *L. brevis* antagonized *C. jejuni* in an *in vitro* model simulating the chicken digestive tract (Chang and Chen, 2000). In another *in vitro* study, a *Lactobacillus*

strain from human origin exerted a bactericidal effect against *Campylobacter*, presumably by organic acid or anti-*Campylobacter* peptide production (Chaveerach et al., 2004b). Two other isolates (*L. salivarius* NRRL B-30514 and *Paenibacillus polymyxa* NRRL-B-30509, 0.2–0.5 ml administered) were not able to reduce *Campylobacter* colonization, but were shown to produce anti-*Campylobacter* bacteriocins (see section 3.6) (Stern et al., 2008). Svetoch and Stern (2010) found hundreds of strains exerting *in vitro* activity against *C. jejuni* by screening thousands of *Bacillus, Paenibacillus, Lactobacillus, Streptococcus, Enterococcus* and *Escherichia* isolates. Administration of 8 $\log_{10}$ cfu/day *Bifidobacterium longum* PCB 133 for 15 days reduced *C. jejuni* numbers to chicks by 1 $\log_{10}$ (Santini et al., 2010). In contrast to the *in vitro* anti-*Campylobacter* activity of *Enterococcus faecalis* MB 5259 (Robyn et al., 2012), daily administration of 4-8 $\log_{10}$ cfu of this strain to broiler chicks did not reduce *C. jejuni* colonization (Robyn et al., 2013).

To stimulate beneficial microbial strains, prebiotic compounds may be added to the broiler feed. To date, only 0.2% mannanoligosaccharide and 0.1% xylanase have yet been shown to significantly reduce cecal *C. jejuni* numbers, by up to 0.5 $\log_{10}$ (Fernandez et al., 2000; Baurhoo et al., 2009).

Competitive exclusion, by stimulating non-pathogenic bacteria that compete for the same niche as *Campylobacter*, may enhance resistance of the microbiome against *Campylobacter* colonization. Administering heterologous *C. jejuni* isolates from chickens to broilers resulted in partial competitive exclusion of human pathogenic *C. jejuni* strains (at least 25% reduction, *C. jejuni* strains administered at a 1:1 ratio, concentrations not specified) (Chen and Stern, 2001). Calderón-Gómez et al. (2009) identified *C. jejuni* strain 331 that was able to replace other strains and was maintained in the digestive tract until slaughter age during three independent trials. Administration of *Citrobacter diversus*, *Klebsiella pneumoniae* and *Escherichia coli*, in several combinations, prevented or reduced *C. jejuni* colonization, which

was enhanced by adding 2.5% mannose to the chicken diet (Schoeni and Wong, 1994). Scupham et al. (2010) identified a subtype I of *Megamonas hypermegale* as a potential competitive exclusion microorganism. The authors inoculated day-old turkeys with cecal contents of *Campylobacter*-free adult turkeys, treated them with different antibiotics to modify these microbial communities and challenged them with *Campylobacter*. Subsequently, it was investigated which microbiota were able to outcompete *Campylobacter*.

Although many studies investigating probiotics, prebiotics and competitive exclusion have been performed, more research is needed before field applications will be possible.

3.6. BACTERIOCINS

Bacteriocins are antimicrobial polypeptides produced by competing microorganisms (Svetoch and Stern, 2010). Bacteriocin producing strains, specifically selected against *Campylobacter*, would have a minimal impact on the gut microbiome (Svetoch and Stern, 2010; Meunier et al., 2016a). Therapeutic administration of 250 mg microcoated bacteriocin/kg of feed from *Lactobacillus salivarius* NRRL B-30514 (Stern et al., 2006), *Paenibacillus polymyxa* NRRL B-30509 (Stern et al., 2005), *Enterococcus durans/faecium/hirae* NRRL B-30745 (BCN E 760 bacteriocin) (Line et al., 2008) or *E. faecium* NRRL B-30746 (BCN E 50-52) (Svetoch et al., 2008) to broiler chicks reduced cecal *Campylobacter* colonization by at least 5 $\log_{10}$.

3.7. BACTERIOPHAGES

Bacteriophages or phages are viruses that infect specific host bacteria (Wagenaar et al., 2005; Hagens and Loessner, 2010; Robyn et al., 2015). *Campylobacter*-specific phages can naturally be found in the chicken gut (Connerton et al., 2011).

Wagenaar et al. (2005) found that administering 9-11 $\log_{10}$ plaque forming units (pfu) phages 69 (NCTC 12669) and 71 (NCTC 12671) initially reduced cecal *Campylobacter* colonization by 2-3 $\log_{10}$ but that bacterial numbers were again increased after five days. In another study, a short-term 2 $\log_{10}$ decrease in cecal *Campylobacter* numbers was also obtained using 7-9 $\log_{10}$ pfu phage CP220 (El-Shibiny et al., 2009). Since bacterial levels seem to recover after a temporary drop, administering a pulse dose before slaughter seems to be the best option. On the contrary, the 2 $\log_{10}$ decrease obtained by Carvalho et al. (2010) was maintained for seven days after administration of 6-7 $\log_{10}$ pfu a phage cocktail (phages phiCcoIBB35, phiCcoIBB37 and phiCcoIBB12) through the feed. Administration of the same phages by oral gavage did not protect the chickens against *Campylobacter* colonization. Also other phages have been used against *Campylobacter*, with varying results (Janež and Loc-Carrillo, 2013; Meunier et al., 2016a).

Phages can also be used for reducing *Campylobacter* numbers on broiler carcasses (Loc Carrillo et al., 2005). Since they need their host for replication, the use of bacteriophages is self-limiting (Janež and Loc-Carrillo, 2013; Meunier et al., 2016a). However, the long-term efficacy of these phages can be questioned because of a high rate of emerging resistance. In the studies above, respectively 2% and 13% of the *Campylobacter* population was shown to be resistant against the phages used (El-Shibiny et al., 2009; Carvalho et al., 2010). In the latter study, 6% of the strains were shown to be resistant before phage application (Meunier et al., 2016a). To bypass these problems, a cocktail of multiple phage strains should be used.

3.8. IMMUNISATION

3.8.1. WHOLE CELL VACCINES AND DERIVED FORMULATIONS

Poultry vaccination is considered one of the most effective strategies to impact human campylobacteriosis (de Zoete et al., 2007). Whole cell vaccines consist of bacterial cells killed by for example heat or formaldehyde treatment, or attenuated bacteria lacking virulence and/or colonization capacity (de Zoete et al., 2007). These vaccines were the first to be used for experimental vaccination against *Campylobacter*. Oral vaccination of broilers with 2 or 3 doses of 9 $\log_{10}$ formalin inactivated *C. jejuni* strain F1BCB reduced cecal *C. jejuni* numbers by 16-93% (Rice et al., 1997). Widders et al. (1998) combined a whole cell vaccine with purified flagellin, obtaining a significant reduction of 2 $\log_{10}$ after two intraperitoneal injections but not after oral administration (vaccination dose not defined).

3.8.2. SUBUNIT VACCINES

Next, researchers started administering protein and protein-derived vaccines, such as whole proteins, protein subunits, peptides, hybrid and fusion proteins, as such, on a carrier or DNA-vectored.

The first experimental anti-*Campylobacter* subunit vaccines were based on flagellin (Table 2). Widders et al. (1998) obtained a systemic and mucosal humoral immune response using purified flagellin (vaccination dose not defined), but this did not result in *in vivo* protection of broilers after challenge with *Campylobacter*. In another study, chickens were vaccinated with 250-1000 µg of a fusion protein of the flagellin subunit A (FlaA) and heat-labile enterotoxin subunit B after which an immune response was induced in some birds and a significant reduction from 49.3% to 27.6% *C. jejuni* colonized birds was obtained (Khoury and

Meinersmann, 1995). More recently, Neal-McKinney et al. (2014) reduced the *Campylobacter* numbers in broilers by 3 $\log_{10}$ cfu/g cecal content after vaccinating twice with 240 µg FlaA. Huang et al. (2010) incorporated FlaA DNA, vectored in the pCAGGS plasmid, in chitosan nanoparticles to enhance mucosal uptake and immunized chickens with 150 µg of the FlaA DNA via intranasal administration. This induced serum IgY and gut mucosal IgA and resulted in a 2-3 $\log_{10}$ cfu/g reduction of cecal and colon *Campylobacter* numbers.

Other antigens have also been used. Buckley et al. (2010) successfully immunized chicks with 14 µg Cja subunit A (CjaA), resulting in an increase in IgY titers and a 1.6-3 $\log_{10}$ reduction of cecal *C. jejuni* numbers. *In ovo* vaccination with GEM particles or liposomes carrying the hybrid protein rCjaAD, CjaA modified with CjaD epitopes, significantly but slightly reduced the cecal numbers of a heterologous *C. jejuni* strain from 10 $\log_{10}$ to resp. 9 and 7 $\log_{10}$ (see also section 3.8.4) (Kobierecka et al., 2016). Outer membrane proteins (OMPs) encapsulated in polylactide-co-glycolide nanoparticles did not protect broilers when administered orally at a dose of 25-250 µg, but a significant immune response and reduction in intestinal colonization under the detection limit was obtained when injecting 125 µg OMPs subcutaneously (Annamalai et al., 2013). Administering CmeC, an outer membrane component involved in *Campylobacter* colonization, both orally (200 µg) and subcutaneously (50-200 µg), induced a serum immune response but did not protect chickens against *C. jejuni* (Zeng et al., 2010). Subcutaneous vaccination with 200 µg recombinant Dps, a protein involved in biofilm formation and colonization, did not protect broiler chicks against *C. jejuni* colonization (Theoret et al., 2012). Vaccination with 20.84 µg FliD resulted in a temporary 2 $\log_{10}$ reduction of *C. jejuni* numbers, in contrast to FspA (Chintoan-Uta et al., 2016). Neal-McKinney et al. (2014) investigated CadF, FlpA and CmeC, proteins involved in chicken colonization, as potential vaccine candidates. Administration of 240 µg CadF and CmeC resulted in serum reactivity, but not in protection, however, 240 µg FlpA on the one hand and

a fusion of these proteins combined with the protein mixture as a booster on the other hand did result in a 3 $\log_{10}$ reduction of cecal *C. jejuni* numbers.

Table 2. Overview of subunit vaccines applied against *Campylobacter* in chickens

Antigen	Dose	Results	Reference
Flagellin, purified	Not defined	Systemic and mucosal humoral immune response, no reduction	Widders et al. (1998)
FlaA and heat-labile enterotoxin subunit B fusion protein	250-1000 µg	Immune response, reduction from 49.3% to 27.6% *C. jejuni* colonized birds	Khoury and Meinersmann (1995)
FlaA, CadF, FlpA, CmeC; fusion protein and protein mixture (booster)	240 µg (vaccinated twice)	Reduction of *Campylobacter* numbers in broilers by 3 $\log_{10}$ cfu/g cecal content (FlaA, FlpA, fusion protein + protein mixture); serum reactivity, no reduction (CadF, CmeC);	Neal-McKinney et al. (2014)
FlaA DNA + chitosan nanoparticles	150 µg	Induction of serum IgY and gut mucosal IgA, 2-3 $\log_{10}$ cfu/g reduction of cecal and colon *Campylobacter* numbers	Huang et al. (2010)
CjaA	14 µg	Increased IgY titers, 1.6-3 $\log_{10}$ cfu/g reduction of cecal *C. jejuni* numbers	Buckley et al. (2010)
rCjaAD, hybrid protein + GEM particles, liposomes	*In ovo* administration, 0.1 mL	Reduction of the cecal numbers of heterologous *C. jejuni* strain from 10 $\log_{10}$ to resp. 9 and 7 $\log_{10}$ cfu/g	Kobierecka et al. (2016)
OMPs, polylactide-co-glycolide nanoparticles	25-250 µg (oral), 125 µg (subcutaneous)	Resp. no reduction, significant immune response and reduction in intestinal colonization under the detection limit	Annamalai et al. (2013)
CmeC	200 µg (oral), 50-200 µg (subcutaneous)	Induction of serum immune response, no reduction	Zeng et al. (2010)
Dps, recombinant	200 µg (subcutaneous)	No reduction	Theoret et al. (2012)
FliD, FspA	20.84 µg	Resp. temporary 2 $\log_{10}$ reduction of *C. jejuni* numbers, no reduction	Chintoan-Uta et al. (2016)

Recently, novel protein and peptide vaccine candidates were identified by two-dimensional gel-electrophoresis, Western blot analysis and mass spectrometric analysis (Shoaf-Sweeney et al., 2008; Kovach et al., 2011; Hermans et al., 2014) or bio-informatics (Meunier et al., 2016b; Mehla and Ramana, 2017) (Table 3) and it might be interesting to investigate their *in vivo* immunogenic and protective potential. Some interesting vaccine candidates are AtpA, CheV, EfTu, GroEL, LivJ and Tig (Hermans et al., 2014). These proteins function as an ATP synthase subunit (AtpA), a chemotaxis protein associated with transmembrane receptors (CheV), an elongation factor translocated to the surface in several bacteria (EfTu), a heat shock protein shown to mediate *Salmonella* adhesion (GroEL), an amino acid transporter (LivJ) and in protein transport (Tig) (Tsugawa et al., 2007; Shoaf-Sweeney et al., 2008; Nieves et al., 2010; Kovach et al., 2011; Lertsethtakarn et al., 2011; Ribardo et al., 2011; Hermans et al., 2014). These proteins are known or suggested to be expressed on the bacterial cell surface (EfTu, GroEL) or known to be associated with the cell membrane (AtpA, CheV, LivJ, Tig) (Tsugawa et al., 2007; Nieves et al., 2010; Lertsethtakarn et al., 2011; Zhang et al., 2012; Hermans et al., 2014) and showed a strong immunoreactivity during other studies (Shoaf-Sweeney et al., 2008; Nieves et al., 2010; Kovach et al., 2011; Ribardo et al., 2011; Bao et al., 2013; Tan et al., 2015).

Table 3. Overview of subunit vaccine candidates identified by Shoaf-Sweeney et al. (2008), Kovach et al. (2011), Hermans et al. (2014), Meunier et al. (2016b) and Mehla and Ramana (2017). Proteins incorporated in the experimental chapters of this thesis are indicated in bold.

Gene/ID	Protein name	Reference
accC-2	Acetyl-CoA carboxylase, biotin carboxylase	Hermans et al. (2014)
aco-2	Aconitate hydratase 2	Kovach et al. (2011)
aspA	Aspartate ammonia-lyase	Hermans et al. (2014)
atpA	**ATP synthase F1, alpha subunit**	**Kovach et al. (2011), Hermans et al. (2014)**
atpB	ATP synthase F1, beta subunit	Kovach et al. (2011)
cadF	Outer membrane fibronectin-binding protein	Shoaf-Sweeney et al. (2008), Kovach et al. (2011)
cas2	Crispr-associated protein Cas2	Kovach et al. (2011)
ccc13826_1437	Hypothetical protein	Kovach et al. (2011)
cheV	**Chemotaxis protein V**	**Hermans et al. (2014)**
chuA	TonB-dependent heme receptor	Meunier et al. (2016b)
cjaA	Putative solute-binding protein (surface antigen)	Shoaf-Sweeney et al. (2008)
cjaC	Solute-binding OMP (surface antigen)	Shoaf-Sweeney et al. (2008), Kovach et al. (2011)
cj0178	Putative TonB-dependent outer membrane receptor	Mehla and Ramana (2017)
cj0530	Putative periplasmic protein	Mehla and Ramana (2017)
cjj81176_0126	Putative lipoprotein	Shoaf-Sweeney et al. (2008)
cjj81176_0128	Hypothetical periplasmic protein	Shoaf-Sweeney et al. (2008)
cjj81176_0164	OMP 85 family	Shoaf-Sweeney et al. (2008)
cjj81176_0586	Hypothetical protein, OMP	Shoaf-Sweeney et al. (2008)
cjj81176_1185	Hypothetical protein, OMP	Shoaf-Sweeney et al. (2008)
cjj81176_1295	Fibronectin type III domain protein	Shoaf-Sweeney et al. (2008)
cjj81176_1525	Tungstate ABC transporter protein	Shoaf-Sweeney et al. (2008)

Table 3. (continued)

Gene/ID	Protein name	Reference
clpX	ATP-dependent Clp protease, ATP-binding subunit	Hermans et al. (2014)
cmeA	Membrane fusion protein, RND efflux system pump	Shoaf-Sweeney et al. (2008)
cmeC	Outer membrane channel protein, RND efflux pump	Shoaf-Sweeney et al. (2008)
cmeD	Outer membrane component of efflux system (multidrug efflux system cmeDEF)	Mehla and Ramana (2017)
	ComEC/Rec2 family protein	Kovach et al. (2011)
ctpA	Putative secreted carboxyl-terminal protease	Hermans et al. (2014)
cysM	O-acetylserine sulfhydrylase B	Hermans et al. (2014)
	Putative Cysteine desulfurase	Hermans et al. (2014)
dapA	Dihydrodipicolinate synthase	Hermans et al. (2014)
	DNA-binding response regulator	Kovach et al. (2011)
dctQ	C4-dicarboxylate transporter protein	Kovach et al. (2011)
efG	Translation elongation factor G	Kovach et al. (2011)
efTu	**Translation elongation factor thermo unstable**	**Kovach et al. (2011), Hermans et al. (2014)**
eno	Phosphopyruvate hydratase	Kovach et al. (2011)
fabF	3-Oxoacyl-[acyl-carrier-protein] synthase 2	Kovach et al. (2011)
fabI	Enoyl-(acyl-carrier-protein) reductase	Hermans et al. (2014)
fbaA	Fructose-bisphosphate aldolase, class II	Kovach et al. (2011)
flaA	Flagellin subunit protein A	Shoaf-Sweeney et al. (2008), Hermans et al. (2014)
flaB	Flagellin subunit protein B	Shoaf-Sweeney et al. (2008), Kovach et al. (2011), Hermans et al. (2014)
flgE	Flagellar hook protein	Hermans et al. (2014)
flgE1	Flagellar hook protein	Meunier et al. (2016b)

Table 3. (continued)

Gene/ID	Protein name	Reference
flgE2	Flagellar hook protein	Shoaf-Sweeney et al. (2008)
flgH	Flagellar basal body L-ring protein	Meunier et al. (2016b)
flgK	Flagellar hook-associated protein	Kovach et al. (2011), Meunier et al. (2016b)
fli	Flagellar motor switch protein	Kovach et al. (2011)
frdA	Fumarate reductase flavoprotein subunit	Kovach et al. (2011)
fumC	Fumarate hydratase	Hermans et al. (2014)
gltX-1	Glutamyl-tRNA synthetase	Kovach et al. (2011), Hermans et al. (2014)
	Glutathionylspermidine synthase family protein	Hermans et al. (2014)
glyQ	Glycyl-tRNA synthetase alpha subunit	Hermans et al. (2014)
groEL	**Co-chaperonin**	**Hermans et al. (2014)**
groL	Chaperonin	Kovach et al. (2011)
grpE	Heat shock protein	Hermans et al. (2014)
guaA	MP synthase (glutamine-hydrolyzing)	Hermans et al. (2014)
hemL	Glutamate-1-semialdehyde-2,1-aminomutase	Hermans et al. (2014)
	High affinity branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	Hermans et al. (2014)
hyfI	Hydrogenase-4 component I	Kovach et al. (2011)
hypD	Hydrogenase expression/formation protein	Hermans et al. (2014)
ilvE	Branched-chain amino acid aminotransferase	Hermans et al. (2014)
ilvH	Acetolactate synthase small subunit	Kovach et al. (2011)
jlpA	Surface-exposed lipoprotein	Meunier et al. (2016b)
livJ	**Branched-chain amino acid ATP-binding cassette transport protein**	**Hermans et al. (2014)**
mapA	Outer membrane lipoprotein	Shoaf-Sweeney et al. (2008)

Table 3. (continued)

Gene/ID	Protein name	Reference
metQ	D-Methionine-binding lipoprotein	Kovach et al. (2011)
	Methyl-accepting chemotaxis protein	Kovach et al. (2011), Hermans et al. (2014)
	Putative methyltransferase	Hermans et al. (2014)
omp	Major outer membrane protein	Hermans et al. (2014)
omp18	Outer membrane protein 18	Kovach et al. (2011)
PEB2	Major antigenic peptide	Shoaf-Sweeney et al. (2008)
PEB3	Major antigenic peptide	Shoaf-Sweeney et al. (2008)
	Peptide transport system substrate-binding protein	Hermans et al. (2014)
	Putative peptidyl-prolyl cis-trans isomerase	Kovach et al. (2011)
pgk	Phosphoglycerate kinase	Hermans et al. (2014)
pldA	Phospholipase A	Meunier et al. (2016b)
porA	Major OMP	Shoaf-Sweeney et al. (2008), Meunier et al. (2016b)
ppa	Inorganic pyrophosphatase	Hermans et al. (2014)
purM	Phosphoribosylformylglycinamidine cyclo-ligase	Hermans et al. (2014)
pyk	Pyruvate kinase	Hermans et al. (2014)
	Radical SAM domain protein	Kovach et al. (2011)
	Response regulator receiver domain protein	Kovach et al. (2011)
sdhB	Succinate dehydrogenase, iron-sulfur protein	Shoaf-Sweeney et al. (2008)
slyD	FKBP-type peptidyl-prolyl cis-trans isomerase	Hermans et al. (2014)
	Succinyl-CoA synthetase beta chain	Hermans et al. (2014)
thiC	Thiamine biosynthesis protein	Kovach et al. (2011)

Table 3. (continued)

Gene/ID	Protein name	Reference
tig	**Trigger factor**	**Kovach et al. (2011), Hermans et al. (2014)**
tktA	Transketolase A	Kovach et al. (2011)
	Transmembrane transport protein	Kovach et al. (2011)
tyrS	Tyrosyl-tRNA ligase	Kovach et al. (2011)
	Putative UDP-glucose 4-epimerase	Hermans et al. (2014)
yaeT	Outer membrane assembly complex	Kovach et al. (2011)
YP 001000153.1	Putative TonB-dependent receptor, degenerate	Meunier et al. (2016b)
YP 001000261.1	Hypothetical protein	Meunier et al. (2016b)
YP 001000437.1	Putative OMP	Meunier et al. (2016b)
YP 001000562.1	Flagellin protein family	Meunier et al. (2016b)
YP 001000945.1	N-Acetylmuramoyl-L-alanine amidase	Meunier et al. (2016b)
YP 999838.1	Hypothetical protein	Meunier et al. (2016b)
YP 999817.1	Hypothetical protein	Meunier et al. (2016b)
	Conserved hypothetical protein	Hermans et al. (2014)

3.8.3. VECTOR-BASED VACCINES

Next to administering the protein itself, a bacterial or viral vector expressing the antigen can also be used (Saxena et al., 2013). The most commonly used vectors for administration of *Campylobacter* antigens are avirulent *Salmonella* mutants. An advantage of this method is that vaccination against both *Campylobacter* and *Salmonella* can be obtained with one vaccine, however there is a risk of the used vector strain regaining virulence (Łaniewski et al., 2014).

Vaccination with 7-8 $\log_{10}$ of a CjaA-expressing *Salmonella* vector induced a specific humoral response and reduced *C. jejuni* numbers in the broiler cecum by 6 or 1.4 $\log_{10}$ (Wyszyńska et al., 2004; Buckley et al., 2010) or ileum by 2 $\log_{10}$ (Layton et al., 2011). On

the contrary, Łaniewski et al. (2014) found no reduction in *C. jejuni* numbers despite inducing specific serum IgY and mucosal IgA antibodies after vaccination with 8 $\log_{10}$ of a CjaA-expressing *Salmonella* vector. The results of these studies varied depending on the *Campylobacter* test strain and vaccination carrier strains used. Oral vaccination of chicks with 7 $\log_{10}$ of a *Salmonella* vector expressing CjaA, CadF, CiaB, cj1496 or a combination of these antigens, resulted in an up to 2-3 $\log_{10}$ reduction of *C. jejuni* numbers when using CjaA, CadF or the combination (Saxena et al., 2013). When using the ACE393 antigen in a *Salmonella* vector (7.4 $\log_{10}$ orally administered), IgY was induced but no significant reduction of ileal *Campylobacter* numbers was found, while using OMP18/CjaD increased serum IgY and mucosal IgA and reduced ileal *Campylobacter* numbers to under the detection limit (Layton et al., 2011). Vaccination with 8 $\log_{10}$ of a *Salmonella* vector carrying a fusion of Peb1A and the tetanus toxin significantly reduced *C. jejuni* numbers by 1.64 $\log_{10}$, but not when the toxin was combined with GlnH or ChuA (Buckley et al., 2010). Oral administration of an attenuated *Salmonella* vector producing Dsp resulted in a significant reduction of cecal *C. jejuni* bacteria by 2.48 $\log_{10}$ (vaccination dose not defined) (Theoret et al., 2012). Finally, also *Eimeria tenella* has been used as a vector for *C. jejuni* CjaA for oral vaccination of broiler chicks with 100-5000 parasites, resulting in a significant reduction of cecal *C. jejuni* numbers by 1 $\log_{10}$ (Clark et al., 2012).

3.8.4. IN OVO VACCINATION

Another method to induce immunity in chickens, is to vaccinate the chick embryo in the egg. This is already applied for Marek's disease, infectious bursal disease, and Newcastle disease (Peebles, 2018). Although the chicken immune system is only fully developed at several weeks after hatching, the 18-day-old embryo is capable of reacting to an administered antigen

(Avakian et al., 2007). Only a few studies have investigated this delivery method against *Campylobacter* yet. Noor et al. (1995) demonstrated a humoral immune response in the serum, intestine, bile and spleen of broilers against *Campylobacter* after *in ovo* vaccination with a heat-killed *C. jejuni* isolate, however, protection against challenge was not investigated. More recently, *in ovo* vaccination with 20-40 µg of a recombinant flagellin-based subunit vaccine with intrinsic adjuvant activity induced serum IgY and IgM, but no mucosal immune response or protection (Radomska et al., 2016). At the same time, Kobierecka et al. (2016) combined the hybrid protein rCjaAD, which is CjaA presenting CjaD epitopes, with Gram-positive enhancer matrix (GEM) particles or liposomes, which resulted in a colonization reduction from 10 $\log_{10}$ to resp. 9 and 7 $\log_{10}$ of a heterologous *C. jejuni* strain. Godlewska et al. (2016) used native and modified *C. jejuni* outer membrane vesicles (OMVs), containing 200 µg protein per vaccination dose, which resulted in a maximum reduction of 1 $\log_{10}$ of cecal *Campylobacter* colonization. Finally, administering 50 µg of a DNA-vaccine based on CmeC or CfrA did not induce IgY or IgA in chicken embryos and consequently no protection against *Campylobacter* was obtained (Liu et al., 2019).

3.8.5. PASSIVE IMMUNISATION

Administering antibodies could be a promising strategy for controlling enteric infections, such as campylobacterioses (Mine and Kovacs-Nolan, 2002; Schade et al., 2005; Chalghoumi et al., 2009; Yegani and Korver, 2010; Gadde et al., 2015; Hedegaard and Heegaard, 2016). The use of specific antibodies was shown to reduce *Campylobacter* colonization in chickens, by pre-incubating the *Campylobacter* inoculum with antibody preparations originating from rabbit antiserum and chicken bile (Stern et al., 1990a; b), or bovine milk and chicken eggs by (Tsubokura et al., 1997) and by therapeutic administration of the latter antibody preparations

(Tsubokura et al., 1997). Maternal antibodies passed along from the hen via the egg yolk were shown to protect young chicks against *Campylobacter* colonization until two to three weeks post-hatch (Sahin et al., 2003b). Eggs of vaccinated hens can contain high levels of specific antibodies and might therefore be a suitable production platform for protective antibodies (Bizanov, 2018).

Five years ago, Hermans et al. (2014) demonstrated that prophylactic administration of 5% hyperimmune egg yolk from layer hens vaccinated against *C. jejuni* through the feed of broilers could reduce *C. jejuni* colonization by approximately 4 $\log_{10}$ compared to control chicks. Moreover, transmission to non-inoculated contact birds was reduced tremendously or completely prevented. At the same time, other researchers developed hyperimmune egg yolk powder (HEYP) against several *C. jejuni* proteins (Al-Adwani et al., 2013), but administration of these HEYPs at 10% through the feed did not protect broilers against *C. jejuni* colonization (Paul et al., 2014). Nanobodies, camelid antibody fragments, targeting flagellin and MOMP were shown to have anti-*Campylobacter* properties *in vitro*, however *in vivo* protection has not been investigated yet (Vanmarsenille et al., 2017, 2018).

3.8.6. WHY IS THERE STILL NO VACCINE AVAILABLE?

The studies described above indicate that immunisation can reduce *Campylobacter* colonization of flocks under experimental conditions. Immunisation is considered to possibly be the most effective measure (de Zoete et al., 2007). However, despite many successful studies, no commercial vaccine is available yet. The development of an industrially applicable vaccine is hampered by the timing of the development of the chicken immune system. The first weeks after hatching, the chicken immune system is not yet fully developed (Friedman et al., 2003; Bar-Shira and Friedman, 2006) and maternal immunity may interfere with

vaccination (Sahin et al., 2003b). Since *Campylobacter* infection may occur at two weeks post hatch (Jacobs-Reitsma et al., 1995; Evans and Sayers, 2000; Sahin et al., 2001, 2003b; Herman et al., 2003; van Gerwe et al., 2009; Cawthraw and Newell, 2010), vaccination cannot be delayed too long. Also, the chicken immune system and the interaction of the bacterium with the chicken host are not completely understood yet. For example, the question if *Campylobacter* sp. is a commensal in chickens or not, still remains unanswered (Humphrey et al., 2014). Manually vaccinating individual chicks, by injection or per os, would be very labour-intensive and time-consuming. Alternatively, mass immunisation by administering the vaccine or *Campylobacter*-specific antibodies through the feed or drinking water, or by automated *in ovo* vaccination, may bypass this problem.

4. Conclusion

Campylobacteriosis remains the most important bacterial foodborne zoonosis in the EU. Since the main source of the infections can be traced back to poultry, controlling the pathogen in primary poultry production will be essential to combat this disease. Despite the fact that many different strategies are being investigated, there are no effective measures yet to limit *Campylobacter* in poultry.

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CHAPTER 2: SCIENTIFIC AIMS

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Campylobacter is the most important bacterial zoonosis worldwide, originating mainly from poultry. It is estimated that a 3 $\log_{10}$ reduction of cecal *Campylobacter* numbers in chickens, corresponding to a 2 $\log_{10}$ reduction of *Campylobacter* numbers on carcasses, would reduce human campylobacteriosis incidence by 85-96%. Currently no efficient and sustainable control measures exist to reduce cecal *Campylobacter* numbers in chickens. Previously, a proof of concept was delivered that passive immunisation of broiler chicks by oral administration of egg yolk antibodies against a whole cell *C. jejuni* vaccine protects the birds against *C. jejuni* colonization.

The scientific aim of this project was to explore the efficacy and applicability of passive and active immunisation against *C. jejuni* and *C. coli* in broilers, using two novel vaccines: a bacterin mix of thirteen genetically diverse *C. jejuni* and *C. coli* strains and a subunit vaccine consisting of six immunodominant *C. jejuni* proteins.

For this purpose, the following research questions were addressed:

1. Does passive immunisation with egg yolk antibodies, directed against the bacterin or subunit vaccine, protect broiler chicks against a diversity of *C. jejuni* and *C. coli* strains, when applied (a) prophylactically or (b) therapeutically?

2. Does lyophilisation of the hyperimmune egg yolks, which would improve feasibility of the fresh yolks, affect the efficacy of passive immunisation?

3. Does active immunisation, more specifically *in ovo* vaccination against the bacterin or subunit vaccine, induce an antibody response in broiler chickens and does it protect the chickens against *Campylobacter* colonization?

CHAPTER 3: EXPERIMENTAL STUDIES

CHAPTER 3: EXPERIMENTAL STUDIES

3.1. Passive immunisation

3.1.1. Reducing *Campylobacter jejuni* colonization in broiler chickens by in-feed supplementation with hyperimmune egg yolk antibodies

3.1.2. Lyophilisation of hyperimmune egg yolk: effect on antibody titer and protection of broilers against *Campylobacter* colonization

3.2. Active immunisation

In ovo vaccination of broilers against *Campylobacter jejuni* using a bacterin and subunit vaccine

3.1. Passive immunisation

3.1.1. Reducing *Campylobacter jejuni* colonization in broiler chickens by in-feed supplementation with hyperimmune egg yolk antibodies

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ABSTRACT

Campylobacter infections sourced mainly to poultry products, are the most important bacterial foodborne zoonoses worldwide. No effective measures to control these infections in broiler production exist to date. Here, we used passive immunisation with hyperimmune egg yolks to confer broad protection of broilers against *Campylobacter* infection. Two novel vaccines, a bacterin of thirteen *Campylobacter jejuni* (*C. jejuni*) and *C. coli* strains and a subunit vaccine of six immunodominant *Campylobacter* antigens, were used for the immunisation of layers, resulting in high and prolonged levels of specific immunoglobulin Y (IgY) in the hens' yolks. In the first *in vivo* trial, yolks (sham, bacterin or subunit vaccine derived) were administered prophylactically in the broiler feed. Both the bacterin- and subunit vaccine-induced IgY significantly reduced the number of *Campylobacter*-colonized broilers. In the second *in vivo* trial, the yolks were administered therapeutically during three days before euthanasia. The bacterin IgY resulted in a significant decrease in *C. jejuni* numbers per infected bird. The hyperimmune yolks showed strong reactivity to a broad representation of *C. jejuni* and *C. coli* clonal complexes. These results indicate that passive immunisation with hyperimmune yolks, especially bacterin derived, offers possibilities to control *Campylobacter* colonization in poultry.

INTRODUCTION

Campylobacteriosis is one of the most important foodborne bacterial diseases worldwide and has been the most commonly reported zoonosis in the EU since 2005 (EFSA, 2017). Clinical symptoms such as fever and diarrhoea are usually self-limiting, although in rare cases complications can occur, leading to reactive arthritis (Hannu et al., 2002), Guillain-Barré syndrome (GBS) (Nachamkin, 2002) and inflammatory bowel disease (IBD) (Neal, 1997). The disease is mainly caused by *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) (EFSA, 2017) and contaminated chicken meat is considered a major source of infection (Humphrey et al., 2007). Worldwide, over 50% of poultry meat is contaminated with *Campylobacter* (Suzuki and Yamamoto, 2009). However, no effective measures to limit *Campylobacter* infections in primary broiler chicken production exist to date (Hermans et al., 2011a). Once a chicken is infected, the pathogen rapidly spreads infecting almost 100% of the flock within a week (Stern et al., 2001).

Interestingly, chickens are only colonized from the age of two to three weeks onwards (Berndtson et al., 1996; van Gerwe et al., 2009), which is presumably due to the protection by maternal IgY antibodies (MAB) (Sahin et al., 2003; Chalghoumi et al., 2009a; Cawthraw et al., 2010). These antibodies are transferred from the serum of the mother to the egg yolk, protecting the chicks during the first weeks when their immune system is not yet fully developed (Chalghoumi et al., 2009a). From two weeks onward, the blood concentration of MAB against *Campylobacter* drops significantly, which coincides with an increased colonization susceptibility of the chickens. As a measure, pure MAB or egg yolks of immunized chickens containing pathogen specific MAB can be added to the feed of the chicks to prolong this effect (Chalghoumi et al., 2009a; Tsubokura et al., 1997). Previously, Hermans et al. (2014) immunized laying hens with a whole cell lysate of *C. jejuni* or its hydrophobic protein fraction, and successfully used their eggs to protect young chickens

against *Campylobacter* infection. As such, passive immunisation of broiler chickens using egg yolk IgY offers possibilities to control *C. jejuni* colonization in broiler flocks.

The vaccines tested by Hermans et al. (2014) were based on one single *C. jejuni* strain, which is not representative for the field situation with many genetically different strains (Duarte et al., 2016). A bacterin containing heterogeneous *Campylobacter* strains might offer a much broader target reactivity. Also, Hermans et al. (2014) identified several immunodominant *C. jejuni* antigens. A subunit vaccine containing a mix of broadly conserved, immunodominant proteins could lead to a well-defined and standardized vaccine.

We developed two vaccines to immunize laying hens against *C. jejuni* and *C. coli* to obtain IgY-rich eggs that confer broad protection of chickens against *C. jejuni* and *C. coli* infection: a bacterin consisting of genetically heterogeneous *Campylobacter* strains relevant to the field situation and a subunit vaccine containing multiple recombinant immunodominant antigens of *C. jejuni* strain KC40 (Hermans et al., 2014). Egg yolks of hens immunized with these vaccines were used for passive oral immunisation of broiler chickens to investigate their prophylactic and therapeutic efficacy against experimental *Campylobacter* infection in broiler chickens. Finally, the reactivity of these egg yolks to a variety of *C. jejuni* and *C. coli* strains, belonging to different clonal complexes was tested as a proxy for the breadth of protection.

METHODS

Experimental animals

Commercial Lohmann Brown-Classic laying hens, LSL-Classic laying hens and Ross 308 broiler chickens of both sexes were purchased at a local hatchery (layers at De Biest, Kruishoutem, Belgium and broilers at Vervaeke-Belavi, Tielt, Belgium). The animals were provided with a commercial feed and water *ad libitum*. Husbandry, experimental procedures,

euthanasia methods and bio-safety precautions were approved by the Ethical Committee (EC) of the Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium (EC number: 2016/28) and in accordance with the relevant guidelines and regulations. Birds were proved to be free of *Campylobacter* by examination of mixed fecal samples using standard methods as described by Hermans et al. (2011a).

Bacterial strains and culture conditions

The *Campylobacter* strains used in this study are listed in Table 1. For all experimental infections in the *in vivo* trials, *C. jejuni* reference strain KC40 from poultry origin was used, which colonizes chickens to a high level (Van Deun et al., 2008). For bacterin composition, *Campylobacter* strains were kindly provided by Dr. Nadine Botteldoorn (Sciensano, Brussels, Belgium), except for the *C. jejuni* KC40 reference strain which was previously isolated at the Flanders Research Institute for agriculture, fisheries and food (ILVO, Melle, Belgium). The strains are from chicken origin and were selected based on their genetic heterogeneity based on multilocus sequence typing (MLST), prevalence ratio in broilers (Duarte et al., 2016) and relatedness to human campylobacteriosis cases (Botteldoorn et al., 2016). The remaining *Campylobacter* strains, used for ELISA cross-reaction studies, are from chicken origin and were selected based on their genetic heterogeneity and distinction from the bacterin strains using MLST (Vinueza-Burgos et al., 2017).

Table 1. *C. jejuni* and *C. coli* strains from chicken origin used in this study.

Campylobacter species	Strain	CC	ST	Origin
C. jejuni	KC40 [b]	677	794	Broiler dunghill
	10kf-1.16 [b]	283	267	Carcass
	7P6.12 [b]	464	464	Feathers
	10C-6.1 [b]	574	305	Ceca
	10kf-4.12 [b]	443	51	Carcass
	10VTDD-8 [b]	UA	905	Unknown
	T124 [b]	658	1044	Ceca
	T84 [b]	354	1073	Ceca
	T70 [b]	21	50	Carcass
	3291 [b]	45	45	Carcass
	5970 [b]	UA	5970	Carcass
	5CT13	48	429	Ceca
	3CT13	52	600	Ceca
	1CT117	257	5742	Ceca
	1CT51	353	462	Ceca
C. coli	2711 [b]	828	854	Carcass
	3250 [b]	UA	5163	Carcass

CC: Clonal complex; ST: Sequence type; [b]: strains incorporated in the bacterin; UA: Unassigned

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 for 17 h under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). *C. jejuni* and *C. coli* bacteria were enumerated by plating tenfold dilutions in Hank's Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen, Carlsbad, CA) 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.

Prevalence and conservation level of immunodominant *Campylobacter* antigens

Based on the results of Hermans et al. (2014), six immunodominant antigens with high reactivity to IgY from eggs of chickens immunized against *C. jejuni* were selected: AtpA, EfTu, GroEL, Tig, CheV and LivJ. These proteins are known or suggested to be expressed on the bacterial cell surface (EfTu, GroEL) or known to be associated with the cell membrane (AtpA, CheV, LivJ, Tig) (Tsugawa et al., 2007; Nieves et al., 2010; Lertsethtakarn et al., 2011; Zhang et al., 2012; Hermans et al., 2014) and showed a strong immunoreactivity during other studies (Shoaf-Sweeney et al., 2008; Nieves et al., 2010; Kovach et al., 2011; Ribardo et al., 2011; Bao et al., 2013; Tan et al., 2015).

The prevalence and the conservation level of the genes coding for these immunodominant proteins were determined in the *Campylobacter* strains selected for constructing the bacterin using PCR. Because of the genetic heterogeneity, separate primers were developed for *C. jejuni* and *C. coli* strains (http://www.ncbi.nlm.nih.gov/gene/) (Table S1, supplementary materials). *Campylobacter* strains were plated on Columbia Sheep Blood agar (CSB, Oxoid)

and incubated overnight at 37°C under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). For DNA extraction, colonies were incubated with 20 µL lysis buffer (1/40 10% SDS, 1/20 1 N NaOH in AquaDest) until the formation of slime was visible, and afterwards incubated at 95°C for 10 min. After cooling to condense the water vapor and short centrifugation, 80 µl high performance liquid chromatography (HPLC, Merck, VWR, Amsterdam, Netherlands) grade water was added. The lysate was centrifuged at 13000 rpm for 5 min and the supernatant was stored at -20°C. The amplification of DNA was performed in a Mastercycler (Eppendorf AG, Hamburg, Germany) in a volume of 25 µL with 1X mastermix [dNTP's, MgCl and NA polymerase of Bioline (Luckenwalde, Germany)] and 0.5 µM of each primer. *C. jejuni* strain KC40 was used as a positive control and blanc HPLC water was added to the mix as a negative control. The PCR program was set at 4 min at 95°C, 35 cycles (1 min at 94°C, 1 min at 57°C, 1 min 30 s at 72°C) and a final elongation step of 15 min at 72°C. The PCR reaction products were analyzed with gel electrophoresis. Sequencing analysis was performed to determine the degree of conservation of the prevalent encoding proteins. For genes consisting of more than 1000 base pairs, multiple primer pairs were developed (Table S1). The DNA amplification and gel electrophoresis were performed as described above. After checking the purity of the bands, sequencing analysis was performed by Eurofins Genomics (Ebersberg, Germany). Data were analyzed using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast) for comparison of the nucleotide sequences, ExPASy Bioinformatics Resource Portal (http://web.expasy.org/translate/) to translate the nucleotide sequences into protein sequences and Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast) for comparison of the protein sequences.

Preparation of recombinant *C. jejuni* antigens

For recombinant production of the immunodominant antigens, derived from the *C. jejuni* reference strain KC40, the *E. coli* Expression System using Gateway® Technology (Invitrogen) was used. Signal peptides in the coding regions, which were screened by using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/), were removed. The coding regions were then amplified by PCR, using Pwo polymerase with proofreading activity (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions and with the primers given in Table S1. The resulting PCR products were cloned into the pENTR™/TEV/D-TOPO® vector (AtpA, EfTu and GroEL) or the pENTR™/SD/D-TOPO® vector (Tig, CheV and LivJ) using the Topo TA cloning kit (Invitrogen) according to the manufacturer's instructions. Next, the genes were transferred into the pDEST™17 destination vector and the resulting expression clones were transformed into BL21-AI One Shot® chemocompetent *E. coli* cells (Invitrogen).

A fresh transformed *E. coli* culture was grown in 100 mL Luria Broth medium (LB, Oxoid) supplemented with 50 µL/mL carbenicillin at 37°C with shaking until an OD_{600} of 0.6-1.0 was reached. The culture was inoculated in 6x 200 mL fresh LB medium supplemented with 50 µL/mL carbenicillin at an OD_{600} of 0.05-0.1 and grown at the same circumstances until an OD_{600} of 0.4 was obtained. Next, 0.2% L-arabinose was added to induce expression of the recombinant antigens. After 6 h of incubation, the cultures were centrifuged (30 min, 4500 rpm) and the pellets were resuspended in binding buffer (40 mM imidazole, 10 mL binding buffer per 1 g pellet). Next, 100 µL lysozyme (20 µg/ml), 200 µl DNase (Sigma Aldrich, Steinheim, Germany), 50 µl 200 x $MgCl_2$ and 100 µl protease inhibitor (Sigma) were added and the mixture was shaken (30 min). After sonication (7x, 15 sec, maximal amplitude), the lysate was centrifuged (30 min, 4500 rpm). The supernatant was purified on Ni-sepharose columns (His GraviTrap; GE Healthcare Bio-science AB, Uppsala, Sweden) according to the

manufacturer's instructions. Bound proteins were eluted with 3 mL elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4) and collected in 17 mL HBSS. The eluate was concentrated to a final volume of 1.5 mL using ultrafiltration (VIVASPIN 20, 5000 MCWO; Sartorius Stedem Biotech, Goettingen, Germany) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Brilliant Blue G-Colloidal (Sigma) coloring and Western blotting.

For the Western blot, separated proteins were electrotransferred from SDS-PAGE gels onto nitrocellulose membranes (Bio-Rad, Nazareth, Belgium) as described previously (Van Steendam et al., 2010). Membranes were blocked in 5% skimmed milk in phosphate buffer saline (PBS) (blocking buffer), incubated overnight with mouse monoclonal antibody to hexahistidine tag (1/3000 in blocking buffer, Icosagen Cell Factory, Tartu, Estonia) at room temperature (RT), rinsed in PBS with 0.3% Tween-20 (wash buffer) and incubated for 1 h at RT with rabbit anti-mouse IgG (whole molecule)–peroxidase antibody (1/30 000 in blocking buffer, Sigma-Aldrich). After a wash step in wash buffer, 10 x CN/DAB Concentrate in Stable Peroxide Substrate Buffer (Thermo Scientific) was added for immunodetection of proteins. Protein patterns were scanned using the GS-800 Calibrated Densitometer (Bio-Rad). The protein concentrations were determined using the *RC DC* Protein Assay (Bio-Rad) and the purified proteins were stored at -80°C until further use.

Bacterin and subunit vaccine preparation

The bacterin was composed as follows: 13 *Campylobacter* strains (Table 1) were grown separately in NB2 until 9 $\log_{10}$ colony forming units (cfu)/mL and killed by overnight incubation with 5 mL 36% formaldehyde/L (Sigma-Aldrich) at 37°C. After centrifugation (30 min at 5000 rpm at 20°C), the pellets were resuspended in 5 mL 36% formaldehyde/L PBS

and incubated overnight at 37°C. After plating on CSB agar and overnight incubation at 37°C to check that all the cells were killed, the suspensions were stored at 4°C. A mix of the 13 *Campylobacter* suspensions was made, so that each bacterin dose consisted of 8.1 $\log_{10}$ cfu inactivated *Campylobacter* (i.e. 7 $\log_{10}$ cfu/*Campylobacter* strain).

For the subunit vaccine, 75 µg protein (i.e. 12.5 µg of each recombinant antigen) was supplemented with HBSS until a volume of 125 µL/vaccine dose. For sham immunisation, 125 µL HBSS was used (negative control).

Each immunisation dose consisted of 250 µL of a 1:1 mixture of the inoculum with Freund's Complete Adjuvant (FCA, Sigma-Aldrich) for the first immunisation and Freund's Incomplete Adjuvant (FIA, Sigma-Aldrich) for the boosters.

Immunisation of layers

Thirty *Campylobacter*-free commercial Lohmann Brown-Classic (LBC) and thirty Lohmann LSL-Classic (LLC) layer hens were assigned to the following immunisation groups at the age of 20 weeks: bacterin (n = 20 LLC hens), subunit (n = 20 LBC hens) and control (n = 10 LLC hens; n = 10 LBC hens). Chickens were immunized by intramuscular injection in the pectoral muscle with the vaccines composed as described above. Three booster immunisations were given in a two-weekly time interval. Starting from one week after the last immunisation, eggs were collected and stored at 4°C.

Determination of egg yolk IgY titers

Campylobacter-specific IgY titers in egg yolks were determined as previously described by Hermans et al. (2014) with minor changes to the protocol. Egg yolks were diluted 1/5 (vol/vol) in HBSS, mixed thoroughly and incubated overnight at 4°C. The supernatant, containing the water-soluble fraction of the egg yolk, was collected for IgY quantification using enzyme-linked immunosorbent assay (ELISA). To determine egg yolk IgY titers against the complete bacterin and the complete subunit vaccine, 96 well flat bottom plates (Nunc MaxiSorp, Nalge Nunc Int., Rochester, NY, USA) were coated (24 h, 4°C) with 10^6 cfu bacterin or 3 µg of a mixture of subunit antigens diluted in 50 µL coating buffer (2.16 g $Na_2CO_3.10H_2O$, 1.935 g $NaHCO_3$ in 500 mL H_2O). To determine egg yolk IgY titers against each recombinant antigen, separately, plates were coated with 3 µg of AtpA, CheV, EfTu, GroEL, LivJ or Tig diluted in 50 µL coating buffer. To determine egg yolk IgY titers against the different *Campylobacter* strains, plates were coated with 10^6 cfu/strain, diluted in 50 µL coating buffer. After washing (3x HBSS, 1x washing buffer: 0,1% Tween-20 in PBS), the wells were blocked (1 h, room temperature) with 100 µl blocking buffer [1% bovine albumin serum (BSA) in washing buffer]. Next, 100 µL of a 1/2 dilution series of the supernatant of the mixed egg yolks was incubated during 60 min at room temperature. Plates were washed as described above and incubated with 100 µL 1/10,000 horseradish peroxidase (HRP)-labelled anti-chicken IgY (Sigma Aldrich) in washing buffer during 90 min at room temperature. After washing as described above, the plates were incubated with 50 µl 3,3',5,5'-tetramethyl benzidine (TMB) substrate (Sigma Aldrich) for 10 min at room temperature in the dark. Next, 50 µL 0.5M H_2SO_4 was added to each well and the absorbance at 450 nm (OD_{450}) was measured using an automated spectrophotometer (Pharmacia LKB Ultrospec III, Gemini BV, Apeldoorn, Nederland). The IgY titers from yolks of immunized hens were reported as the

highest dilution where the OD_{450} was greater than the OD_{450} + three standard deviations of wells containing yolk originating from sham vaccinated birds (Hermans et al., 2014).

Prophylactic efficacy of in-feed supplementation of bacterin and subunit vaccine derived hyperimmune egg yolk on transmission of and cecal colonization with *C. jejuni* in broilers

In trial 1, 81 day-of-hatch *Campylobacter* free broilers were raised in three randomly assigned treatment groups (n = 27/group) and housed in separate isolation units. From the day of hatch until the end of the experiment, the chicks were provided with feed containing 5% (wt/wt) egg yolk (mixed manually through the feed) from hens immunized with the bacterin (group 1), subunit vaccine (group 2) or sham-immunized with HBSS (group 3). 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 10 days of age, the chicks of each group were randomly assigned to three subgroups (n = 9/subgroup) and housed in separate isolation units. At 11 days of age, three seeder chicks of each subgroup were randomly selected and orally inoculated with approximately 1 x 10^5 cfu of *C. jejuni* strain KC40. The birds that were not inoculated are referred to as contact animals or sentinels. Using this model, the *Campylobacter* infection will spread from the seeders to the other animals of the same group reproducing the natural way of infection in the stable and prevention of infection and transmission can be investigated (Hermans et al., 2014). At day 16, all animals were euthanized by injection of an overdose (100 mg/kg) sodium pentobarbital (Kela, Hoogstraten, Belgium) in the wing vein and the cecal content was collected for *C. jejuni* enumeration (as described below).

Therapeutic efficacy of in-feed supplementation of bacterin and subunit vaccine derived hyperimmune egg yolk on cecal *C. jejuni* colonization in broilers

In trial 2 a therapeutic model was used to test the effect of treatments in birds already colonized with *Campylobacter*. For this, 81 day-of-hatch *Campylobacter* free broilers were raised in three randomly assigned groups (n = 27/group) and housed in separate isolation units. At 9 days of age, the chicks of each group were randomly assigned to three subgroups (n = 9/subgroup) and housed in separate isolation units. At 10 days of age, all chicks were orally inoculated with approximately 1 x 10^5 cfu of *C. jejuni* strain KC40, similar to the inoculation during the first trial. From day 19 to 21, the chicks were provided with feed containing 5% (wt/wt) egg yolk (mixed manually through the feed) from hens immunized with the bacterin (subgroups 1, 2, 3), subunit vaccine (subgroups 4, 5, 6) or sham-immunized with HBSS (subgroups 7, 8, 9). Since the therapeutic effect on colonized broilers was to be investigated, all birds were inoculated and sufficient time was given between inoculation and the beginning of the treatment to obtain high *Campylobacter* numbers in the gut, comparable to the field situation. At day 22, all animals were euthanized (as described above) and the cecal content was collected for *C. jejuni* enumeration (as described below).

Cecal *Campylobacter jejuni* enumeration

Cecal contents were weighed and diluted 1:9 (wt/vol) in NB2 with supplements. A 10-fold dilution series was made in HBSS and 100 µl of each dilution was spread on mCCDA plates. Colonies were counted after 24 h and 48 h incubation at 42°C under microaerobic conditions. The diluted samples in NB2 were incubated overnight at 42°C under microaerobic conditions for enrichment. Samples were plated on mCCDA and further incubated. After 24 h and 48 h, the plates were examined for the presence or absence of *C. jejuni*. Samples negative after

titration and enrichment were considered to be free of *Campylobacter* ($<10^2$ cfu/g cecal content, limit of detection). Samples negative after titration but positive after enrichment were considered to contain 10^2 cfu/g cecal content.

Statistical analysis

Data of the *in vivo* trials were analyzed using R 3.3.1. Before statistical analysis, *C. jejuni* numbers were transformed to $\log_{10}$ numbers. The colonization data were analyzed using a hurdle model (Cragg, 1971; Mullahy, 1986), a class of model that assumes that the data are generated by two processes. First, the event that an individual is colonized (i.e. returning a non-zero count) follows a Bernoulli distribution. Given colonization, its intensity or load is a random variable following a discrete or continuous distribution; in this case, a gamma distribution was assumed.

The influence of treatment was assessed by specifying predictors for the Bernoulli probability of occurrence (i.e. probability of colonization, modelled as a logistic function of covariates) and the rates of the gamma distribution (average *C. jejuni* numbers given colonization, modelled as a log-linear function of covariates). In both functions treatment was included as a categorical covariate (bacterin/subunit/control). The sample size prevented the inclusion of an additional covariate for individual type (seeder/sentinel) and the associated interaction term for the first *in vivo* trial. Instead, the analysis was repeated for all birds and for seeders and sentinels separately. A random effect was included at the subgroup (pen) level to account for clustering.

The model was implemented in a Bayesian framework using JAGS (Plummer, 2005). Uninformative, flat priors were used for all parameters. Over three Markov chains, 100.000 iterations were run, discarding the first 50.000 as a burn-in. Convergence was assessed by

visual inspection of the chain histories and using the Gelman-Brooks-Rubin statistic (Brooks and Gelman, 1998). The model was used to estimate the probability of *C. jejuni* colonization and the mean *C. jejuni* numbers in the cecal content of colonized birds for each treatment level. Next, the pairwise differences between those, and the proportion of the respective posterior distributions that had the same sign as the mean were calculated. If working in a null-hypothesis significance testing framework, this can be interpreted as a one-sided test (broilers treated with bacterin-induced antibodies versus control birds, broilers treated with subunit vaccine-induced antibodies versus control birds), estimating the probability that the true difference between treatments is zero or greater (if negative) or smaller (if positive), and thus the level of confidence that the null hypothesis can be rejected. The broilers treated with bacterin- and subunit vaccine-induced antibodies were compared with the equivalent of a two-sided test; the null hypothesis was retained when the posterior distribution of the difference did not encompass zero between the 2.5% and 97.5% quantiles.

RESULTS

Immunodominant antigens are highly prevalent and highly conserved in *C. jejuni*

A PCR analysis, amplifying AtpA, EfTu, GroEL, Tig, CheV and LivJ encoding gene fragments, resulted in positive PCR products in every *C. jejuni* strain screened. Sequence analysis of the PCR products and translation of the nucleotide sequences into protein sequences showed conservation levels of 97% - 100% for both gene and protein sequences (sequence data published elsewhere, Garmyn et al., 2016). Screening the *C. coli* strains, positive PCR products were only obtained for LivJ, CheV and EfTu with conservation levels of 80%, 96% and 99%, respectively, for both gene and protein sequences (sequence data published elsewhere, Garmyn et al., 2016).

Preparation of recombinant *C. jejuni* antigens

Gene copies of *C. jejuni* KC40 AtpA, EfTu, GroEL, Tig, CheV and LivJ were cloned successfully in an entry vector and the pDEST™17 destination vector and expressed in BL21-AI One Shot® *E. coli* transformants. Results of the SDS-PAGE analysis of recombinant *C. jejuni* antigens are shown in Figure 1. All proteins were detected at their corresponding length.

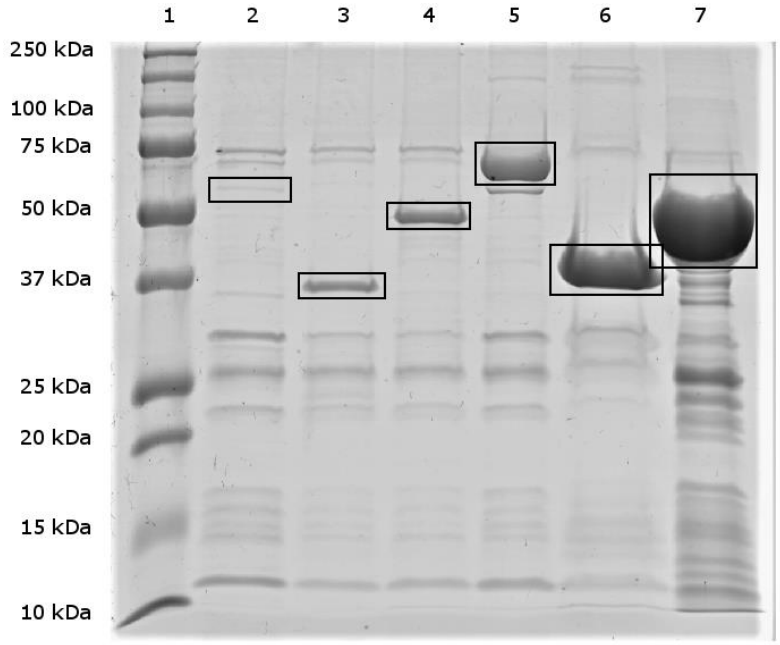

Figure 1. SDS-PAGE analysis visualized by Brilliant Blue G-Colloidal coloring of recombinant *C. jejuni* proteins. Column 1: protein marker with size labelling in kilodalton (kDa) at the left, 2: AtpA (54.8 kDa), 3: CheV (35.8 kDa), 4: EfTu (43.6 kDa), 5: GroEL (58.0 kDa), 6: LivJ (40.1 kDa), 7: Tig (51.0 kDa).

Immunisation of layers with the bacterin and subunit vaccine dramatically induces *Campylobacter*-specific egg yolk IgY titers

The bacterin- and subunit vaccine-induced *Campylobacter*-specific IgY titers in the egg yolks, determined by ELISA, are given in Table 2 and Table 3. These yolk titers were maintained for at least two years after final immunisation.

The bacterin-induced IgY titers against the bacterin and the different *Campylobacter* bacterin strains were all 1:65,536. Also against the *Campylobacter* strains belonging to different clonal complexes (CC) than the bacterin *Campylobacter* strains, IgY titers were remarkably high (1:32,768 to 1:65,536). The subunit vaccine-induced IgY titer against the bacterin was 1:16,384. The subunit vaccine-induced IgY titers against the different *Campylobacter* bacterin strains varied from 1:65,536 (10kf-4.12, T84, T70) to 1:512 (10kf-1.16). For the *Campylobacter* strains belonging to different CC's than the bacterin *Campylobacter* strains, a strong reaction was observed for one strain only (1:4,096; 5CT13). For the subunit vaccine-induced IgY antibodies, a titer of 1:65,536 was obtained against the subunit vaccine and titers of 1:32,768 against each recombinant antigen, separately. The bacterin-induced IgY antibodies showed a much lower reaction, with titers varying from 1:512 (AtpA, EfTu) to non-detectable (< 1:32; CheV, GroEL) against the separate recombinant antigens and a titer of 1:256 against the subunit vaccine.

Table 2. Bacterin- and subunit vaccine-induced egg yolk IgY titers against the bacterin and individual *Campylobacter* strains used in this study, as determined by ELISA.

Strain	Antibody titers induced by	
	Bacterin	**Subunit**
Bacterin	1:65,536	1:16,384
KC40 [b]	1:65,536	1:16,384
10kf-1.16 [b]	1:65,536	1:512
7P6.12 [b]	1:65,536	1:16,384
10C-6.1 [b]	1:65,536	1:16,384
10kf-4.12 [b]	1:65,536	1:65,536
10VTDD-8 [b]	1:65,536	1:16,384
T124 [b]	1:65,536	1:16,384
T84 [b]	1:65,536	1:65,536
T70 [b]	1:65,536	1:65,536
3291 [b]	1:65,536	1:2,048
5970 [b]	1:65,536	1:16,384
2711 [b]	1:65,536	1:16,384
3250 [b]	1:65,536	1:32,768
5CT13	1:65,536	1:4,096
3CT13	1:32,768	<1:32
1CT117	1:32,768	1:32
1CT51	1:32,768	<1:32

[b]: strains incorporated in the bacterin

Table 3. Bacterin- and subunit vaccine-induced egg yolk IgY titers against the subunit vaccine and its individual antigen compounds, as determined by ELISA.

Antigen	Antibody titers induced by	
	Bacterin	**Subunit**
Subunit	1:256	1:65,536
AtpA	1:512	1:32,768
CheV	<1:32	1:32,768
EfTu	1:512	1:32,768
GroEL	<1:32	1:32,768
LivJ	1:128	1:32,768
Tig	1:128	1:32,768

Prophylactic passive immunisation of broilers with bacterin and subunit vaccine derived hyperimmune egg yolk significantly reduces the number of *C. jejuni* colonized birds

In the first *in vivo* trial, the prophylactic effect of hyperimmune egg yolks from immunized laying hens administered to the feed of broiler chickens was investigated. *C. jejuni* numbers per gram (g) cecal content after euthanasia of the chickens are summarized in Table 4. Posterior distributions of the estimated probabilities of *C. jejuni* colonization and mean colonization load are added as Supplemental figure S1. Both the number of *Campylobacter*-positive birds and the mean *C. jejuni* numbers of these positive birds should be considered when interpreting the data about the global *Campylobacter* populations.

Table 4. Number of positive birds and mean cecal *C. jejuni* numbers of colonized broilers receiving standard feed supplemented with 5% (wt/wt) egg yolk from either bacterin-immunized, subunit vaccine-immunized or sham-immunized (control) layers, from day 1 until day 16 (the day of euthanasia). At 11 days of age, 3 seeder birds per group were inoculated with approximately 10^5 cfu *C. jejuni* KC40. A random effect was included in the statistical model at the subgroup (pen) level to account for clustering.

	Number of positive birds			**Mean *C. jejuni* numbers of positive birds (log_{10}(cfu/g cecal content)) (*Standard deviation*)**		
	Bacterin	**Subunit**	**Control**	**Bacterin**	**Subunit**	**Control**
All birds						
Group 1	2/9	4/9	5/9	4.48 (*0.25*)	3.64 (*2.18*)	3.81 (*1.53*)
Group 2	2/9	4/9	8/9	3.50 (*0.71*)	5.71 (*1.83*)	4.31 (*1.74)*
Group 3	0/9	4/9	8/9	- (-)	5.08 (*1.88*)	4.59 (*1.80*)
Treatment	**4/27[a]**	**12/27[a]**	**21/27[b]**	**3.99[a] (*0.71*)**	**4.81[a] (*2.00*)**	**4.30[a] (*1.66*)**
Seeders						
Group 1	0/3	3/3	3/3	- (-)	3.85 (*2.62*)	4.39 (*1.60*)
Group 2	1/3	2/3	3/3	4.00 (-)	6.01 (*2.39*)	5.76 (*2.09*)
Group 3	0/3	1/3	3/3	- (-)	5.04 (-)	4.81 (*2.31*)
Treatment	**1/9[a]**	**6/9[a]**	**9/9[b]**	**4.00[a] (-)**	**4.77[a] (*2.24*)**	**4.99[a] (*1.85*)**
Sentinels						
Group 1	2/6	1/6	2/6	4.48 (*0.25*)	3.00 (-)	2.94 (*1.33*)
Group 2	1/6	2/6	5/6	3.00 (-)	5.41 (*2,00*)	3.45 (*0.81*)
Group 3	0/6	3/6	5/6	- (-)	5.10 (*2.30*)	4.46 (*1.72*)
Treatment	**3/18[a]**	**6/18[ab]**	**12/18[b]**	**3.98[a] (*0.87*)**	**4.85[a] (*1.94*)**	**3.79[a] (*1.38*)**

The total number of *C. jejuni* colonized broilers in the groups receiving hyperimmune egg yolk from bacterin-immunized layers (4/27) and subunit vaccine-immunized layers (12/27) was significantly lower than the number of *C. jejuni* colonized birds in the control subgroups (21/27; resp. $p = 0.0030$ and $p = 0.041$), or a reduction from 78% to resp. 15% and 44% infected birds. This also applies for the seeder birds separately (resp. 1/9, 6/9 and 9/9; $p = 0.00056$ and $p = 0.025$). For the sentinels, hyperimmune egg yolk from bacterin-immunized layers but not from subunit vaccine–immunized layers significantly reduced the number of *C. jejuni* colonized broilers compared to the control broilers (resp. 3/18, 6/18, 12/18; $p = 0.022$ and $p = 0.088$). The treatments did not significantly differ from each other. No significant differences were observed for the *C. jejuni* numbers in birds positive for colonization.

Therapeutic passive immunisation of broilers with bacterin derived hyperimmune egg yolk significantly reduces cecal *C. jejuni* numbers

In the second *in vivo* trial, the therapeutic potential of hyperimmune egg yolk from immunized laying hens administered to the feed of broiler chickens to reduce cecal *C. jejuni* colonization was assessed. *C. jejuni* numbers per g cecal content after euthanasia of the chickens are summarized in Table 5. Posterior distributions of the estimated probabilities of *C. jejuni* colonization and mean colonization load are added as Supplemental figure S2.

Table 5. Number of positive birds and mean cecal *C. jejuni* numbers of colonized broilers receiving standard feed supplemented with 5% (wt/wt) egg yolk from either bacterin-immunized, subunit vaccine-immunized or sham-immunized (control) layers, from day 19 until day 21 (the day of euthanasia). At 10 days of age, all birds were inoculated with approximately 10^5 cfu *C. jejuni* KC40. A random effect was included in the statistical model at the subgroup (pen) level to account for clustering.

	Number of positive birds			**Mean *C. jejuni* numbers of positive birds (log_{10}(cfu/g cecal content)) (*Standard deviation*)**		
	Bacterin	**Subunit**	**Control**	**Bacterin**	**Subunit**	**Control**
Group 1	7/9	9/9	7/9	3.12 (*1.31*)	5.56 (*1.28*)	5.64 (*1.80*)
Group 2	8/9	9/9	7/9	4.08 (*1.59*)	5.19 (*1.84*)	4.96 (*2.09*)
Group 3	7/9	8/9	6/9	4.74 (*1.03*)	5.55 (*1.34*)	5.10 (*2.11*)
Treatment	**22/27[a]**	**26/27[a]**	**20/27[a]**	**4.00[a] (*1.44*)**	**5.43[b] (*1.46*)**	**5.24[b] (*1.67*)**

Most of the broilers were colonized with *C. jejuni* and significant differences were not observed concerning the number of colonized animals between groups. Considering *C. jejuni* numbers in the colonized animals, mean cecal *C. jejuni* numbers in birds receiving hyperimmune egg yolk from bacterin-immunized layers were significantly reduced compared to birds receiving hyperimmune egg yolk from subunit vaccine-immunized layers and control broilers (resp. 4.00, 5.43 and 5.24 log_{10} cfu/g cecal content; p_{eq} = 0.015, p = 0.041). In birds receiving hyperimmune egg yolk from subunit vaccine-immunized layers, mean cecal *C. jejuni* numbers were not reduced compared to the control birds.

DISCUSSION

Passive immunisation of broilers with hyperimmune egg yolk has previously been shown effective at reducing cecal *Campylobacter* loads when the layer hens were immunized using a whole cell lysate or its hydrophobic protein fraction (Hermans et al., 2014). In our study, a bacterin and subunit vaccine were developed for the immunisation of the hens. The bacterin was composed of genetically heterogeneous *C. jejuni* and *C. coli* strains, as these two species are responsible for up to 99.6% of human campylobacteriosis cases in the EU (EFSA, 2017). For the subunit vaccine, proteins were selected based on the reactivity of IgY from *C. jejuni* KC40 immunized layer hens (Hermans et al., 2014), their association with the bacterial cell membrane (Tsugawa et al., 2007; Nieves et al., 2010; Lertsethtakarn et al., 2011; Zhang et al., 2012; Hermans et al., 2014) and previous positive results of vaccination studies (Shoaf-Sweeney et al., 2008; Nieves et al., 2010; Kovach et al., 2011; Ribardo et al., 2011; Bao et al., 2013; Tan et al., 2015). These proteins function as an ATP synthase subunit (AtpA), a chemotaxis protein associated with transmembrane receptors (CheV), an elongation factor translocated to the surface in several bacteria (EfTu), a heat shock protein shown to mediate *Salmonella* adhesion (GroEL), an amino acid transporter (LivJ) and in protein transport (Tig) (Tsugawa et al., 2007; Shoaf-Sweeney et al., 2008; Nieves et al., 2010; Kovach et al., 2011; Lertsethtakarn et al., 2011; Ribardo et al., 2011; Hermans et al., 2014). In this study, the antigens proved to be highly prevalent and conserved in *C. jejuni*. Both vaccines could therefore be expected to offer protection against a broad range of *Campylobacter* strains *in vivo*. Immunisation of hens with these vaccines resulted in a high and specific immune response, comparable to the titers obtained by Hermans et al. (2014). The prolonged response is an economic advantage since the hens would not need to be revaccinated during the production period.

When administered prophylactically, both treatments significantly decreased the number of *C. jejuni* colonized birds, particularly the bacterin-induced antibodies reduced the overall colonization rate from 78% to 15% infected chickens. The subunit vaccine treatment resulted in a reduction of the overall colonization rate to 44% infected birds. When administered therapeutically, which would be cheaper to apply in practice, the treatments were not able to significantly reduce the number of colonized birds, but the bacterin-induced antibodies were capable of significantly reducing *Campylobacter* loads in colonized animals, whereas the subunit-induced antibodies did not. These findings indicate that the bacterin-induced egg yolk antibodies yielded better results than the subunit vaccine-induced antibodies in both *in vivo* trials. Since both vaccines induced a high immune response in laying hens, the difference in protection between both vaccines cannot be attributed to insufficient antibody titers in the subunit yolks. A plausible explanation is that the bacterin contains whole cells and therefore many possible epitopes, while the subunit vaccine only contains the six selected antigens and thus a more limited number of epitopes. Bacterin-induced antibodies were previously shown to protect against *Salmonella* Enteritidis (Fulton et al., 2002; Gürtler et al., 2004) and *Eimeria* sp. (Lee et al., 2009a,b), but Wilkie et al. (2006) found no protective effect against *Clostridium perfringens*. On the contrary, earlier subunit vaccine-induced antibodies failed at protecting against *C. jejuni*, using CadF, FlaA, MOMP, FlpA and CmeC (Paul et al., 2014), and *Salmonella* spp., using outer membrane proteins (Chalghoumi et al., 2009b).

The reduction in cecal *C. jejuni* numbers after therapeutic administration implies that the antibodies must be active in the ceca, since the ceca were already colonized before starting the treatment. However, the site of action may not be restricted to one single gut region. Prophylactic administration possibly allows capturing the bacteria before cecal colonization, which could explain why the prophylactic model resulted in a better overall colonization reduction. Prophylactic and therapeutic passive immunisation experiments with MAB carried

out by Tsubokura et al. (1997) led to resp. a >99% and a 80-95% colonization reduction, also indicating an added value of prophylactic compared to therapeutic administration.

During colonization, *Campylobacter* can be found in the mucus layer (Beery et al., 1988), its site of multiplication, and epithelial cells (Knudsen et al., 2006), hiding from mucosal clearance (Van Deun et al., 2008). Hermans et al. (2014) demonstrated that binding of *Campylobacter* to chicken intestinal mucus was enhanced by specific IgY. The increased bacterial uptake in the mucus layer may promote mucosal clearance, leading to the reduced colonization rates observed in our experiments.

Cross-protection for *Campylobacter* serotypes is one of the major research questions for vaccine development against *Campylobacter*, as formulated by de Zoete et al. (2007). The bacterin-induced antibodies strongly reacted to every bacterin and non-bacterin strain, as determined by ELISA. This indicates that passive immunisation might protect against the other bacterin strains and suggests a possible cross-protection against heterologous *Campylobacter* strains, although this should be confirmed *in vivo*. Nevertheless, these antibodies seem promising at targeting a broad range of *Campylobacter* strains. In contrast, the subunit vaccine-induced antibodies reacted to the bacterin strains but only to one of the non-bacterin isolates. This can have multiple causes: (1) the genes for the subunit proteins may not be present in these strains, (2) the genes might be present but not expressed or show only a low expression rate (Snyder et al., 2013) or (3) the epitopes recognized by the antibodies might be absent or inaccessible (Bagnoli and Rappuoli, 2011). *In vivo* protection against these strains using the subunit vaccine-derived antibodies is very unlikely, strengthening the added value of using the bacterin compared to the subunit vaccine.

In this proof of concept study, only young chickens were included in the experiments. The authors acknowledge that additional studies, including experiments in older chickens until slaughter age and field trials should be performed to support our preliminary findings.

In conclusion, two vaccines, a bacterin consisting of thirteen *C. jejuni* and *C. coli* strains and a subunit vaccine consisting of six immunodominant *Campylobacter* antigens, were developed for the immunisation of laying hens. Administration of hyperimmune egg yolks induced by these vaccines to the feed of broilers, leads to a reduction of infected birds when used prophylactically and a decrease in *Campylobacter* numbers when used therapeutically. Using one of both strategies, the bacterin treatment resulted in the greatest reduction. Although further research will be needed to provide a treatment protocol fully applicable in the industry, our results indicate that passive immunisation of broilers with hyperimmune egg yolks of hens immunized with one of these vaccines, especially the bacterin, offers possibilities to control *Campylobacter* colonization in poultry.

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SUPPLEMENTARY NOTES

We have developed two novel vaccines, a bacterin and subunit vaccine, and used the vaccines to protect broiler chickens against *Campylobacter* colonization by passive immunisation. Posterior distributions from the statistical analysis of the *in vivo* trials' results and primers used for vaccine development can be found in the supplementary materials.

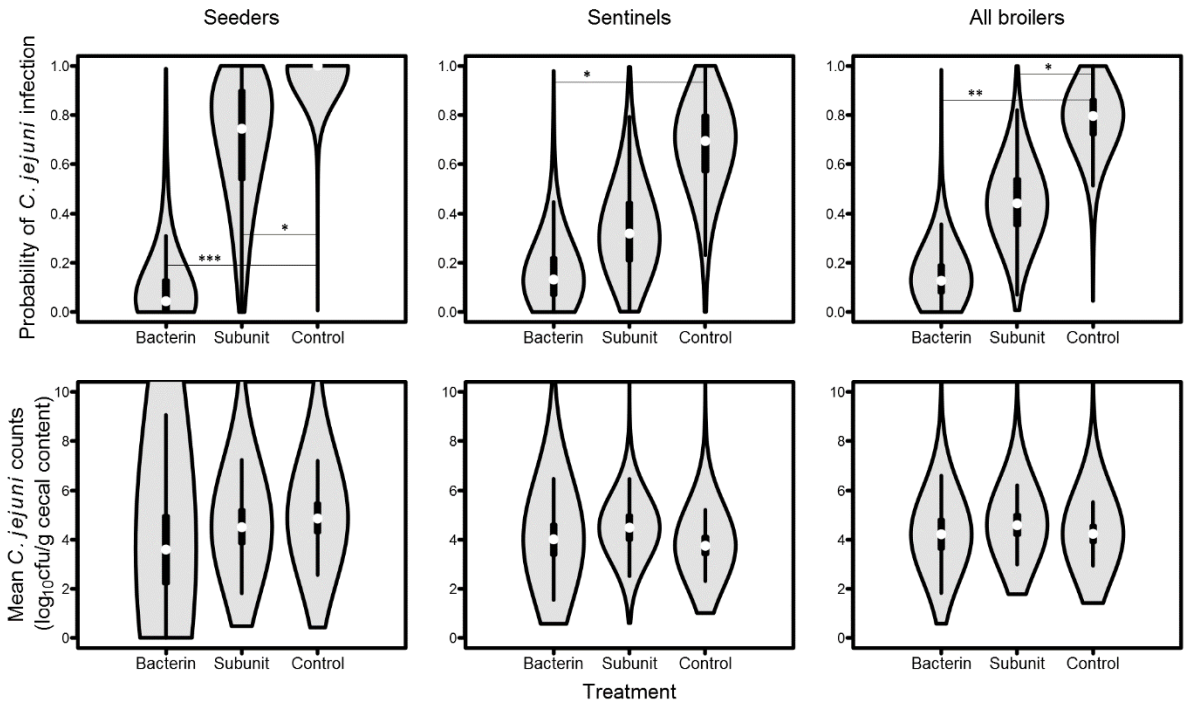

Figure S1. Posterior distributions of the estimated probabilities of *C. jejuni* colonization (top) and mean *C. jejuni* numbers in the cecal content of colonized birds (bottom) for seeders, sentinels or all broilers after prophylactic treatment. The distributions are visualized by a boxplot and Gauss curve. The birds received standard feed supplemented with 5% (wt/wt) egg yolk from either bacterin-immunized (Bacterin), subunit vaccine-immunized (Subunit) or sham-immunized (Control) layers, from day 1 until day 16 (the day of euthanasia). At 11 days of age, seeder birds were inoculated with approximately 10^5 cfu *C. jejuni* KC40.

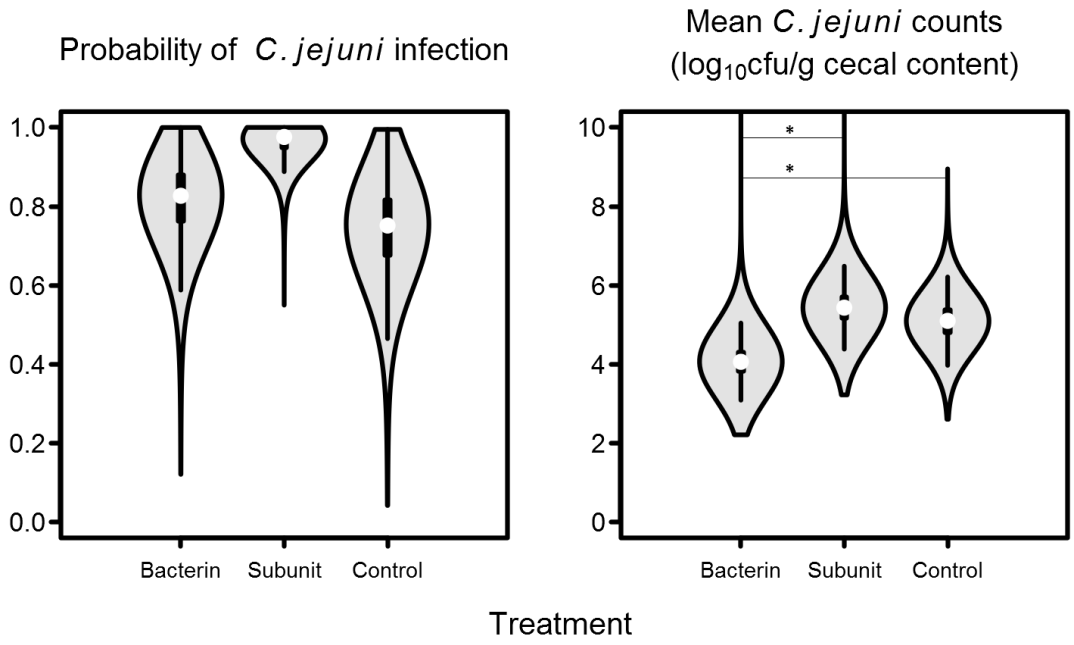

Figure S2. Posterior distributions of the estimated probabilities of *C. jejuni* colonization (left) and mean *C. jejuni* numbers in the cecal content of colonized birds (right) after therapeutic treatment. The distributions are visualized by a boxplot and Gauss curve. The birds received standard feed supplemented with 5% (wt/wt) egg yolk from either bacterin-immunized (Bacterin), subunit vaccine-immunized (Subunit) or sham-immunized (Control) layers, from day 19 until day 21 (the day of euthanasia). At 10 days of age, all birds were inoculated with approximately 10^5 cfu *C. jejuni* KC40.

Supplementary table S1. Primers used for screening of the *Campylobacter* bacterin strains for the presence of immunodominant antigens, sequencing of the prevalent immunodominant antigens and production of blunt end PCR products of the immunodominant antigens

Gene fragment	Forward primer	Reverse primer	Fragment length (bp)
AtpA			
Presence in *C. jejuni*	5'-AGCTGATGAGATCAGTTC-3'	5'-AGCGGAGAATAAGGTGGTTG-3'	1271
Presence in *C. coli*	5'-AGCTGATGAAATCAGTTC-3'	5'-AGTGGAGAATAAGGAGGTTG-3'	1271
Sequencing fragment 1	5'-GAAAGCTCTTCAAAATAAGC-3'	5'-AGACATTTCGCGATAAGC-3'	884
Sequencing fragment 2	5'-GCTCCATATACTGGTGTAACC-3'	5'-CTTTTAGCTGC TTCTTCTGC-3'	925
Production of blunt end PCR products	5'-CACCATGAAATTTAAAGC-3'	5'-TTATAAATGATTTGCTTTAAACTC-3'	1505
CheV			
Presence in *C. jejuni*	5'-ATGGAGCTTGTCGATTTCCG-3'	5'-TTACCCCTGTTCTTGAGATTG-3'	915
Presence in *C. coli*	5'-ATGGAGCTTGTCGATTTCCG-3'	5'-TTACCCCTGTTCTTGAGATTG-3'	915
Sequencing fragment	5'-TGGGTATGTGAAGGACAC-3'	5'-GTCGATTTGCTCAACAAGC-3'	1183
Production of blunt end PCR products	5'-CACCATGTTTGATGAAAATATCG-3'	5'-TTACCCCTGTTCTTGAGATTG-3'	957
EfTu			
Presence in *C. jejuni*	5'-CACGTAATAAGCCACACG-3'	5'-ACCACCTTCACGAATAGC-3'	1138
Presence in *C. coli*	5'-CACGTAATAAGCCACATG-3'	5'-ACCACCTTCACGGATAGC-3'	1138
Sequencing fragment 1	5'-TTTCTGAGCGCTCGTATGGC-3'	5'-CCTGTAACAACAGTACCACG-3'	855

Sequencing fragment 2	5'-CTCTTATGATTTCCCAGGCG-3'	5'-TATTGCCCTAAGGGCGCAAGC-3'	955
Production of blunt end PCR products	5'-CACCATGGCTAAAGAAAA-3'	5'-TTATTTAATAATTTTAGAAAC-3'	1199
GroEL			
Presence in *C. jejuni*	5'-CAGGCGATGGAACAACTACTGC-3'	5'-CCATACCGCTCATATCTGGC-3'	1356
Presence in *C. coli*	5'-CAGGCGATGGAACAACTACTGC-3'	5'-CCATACCGCTCATATCTGGC-3'	1356
Sequencing fragment 1	5'-GCTAAATACGGTGGAACAG-3'	5'-CGCTATATCTTCAAGCATAGC-3'	983
Sequencing fragment 2	5'-CGCTGAAGATATTGAAGG-3'	5'-GAGGATTTGGTATAGGGC-3'	1047
Production of blunt end PCR products	5'-CACCATGGCAAAAGAAAT-3'	5'-TTACATCATTCCGCCCATGC-3'	1637
LivJ			
Presence in *C. jejuni*	5'-CTTTAACTGGAACTGTGGCAGC-3'	5'-GCATTACCGCTTTCATCTATGC-3'	961
Presence in *C. coli*	5'-CATTAACAGGACCAGTGGCTGC-3'	5'-GCATTACCGCTTGCATCAATGC-3'	961
Sequencing fragment 1	5'-GCAGTTCTACAACAGCTTCT-3'	5'-TTGTTAACACCATCTCCTGC-3'	949
Sequencing fragment 2	5'-GCTCCAGTGGCATCTGGAGATA-3'	5'-TGTCCATATGCTGCAGTAGC-3'	902
Production of blunt end PCR products	5'-CACCATGAAGGATATTAATATAGG-3'	5'-TTATGGATTTATAATTGTTTTATA-3'	1115
Tig			
Presence in *C. jejuni*	5'-GGCAAAGCAACTAGATTCTG-3'	5'-TCACAGCAGGCAAAGCTCCTTG-3'	1226
Presence in *C. coli*	5'-AGCAAAGCAATTAGACTCTG-3'	5'-TCACAGCAGGCAAAGCTCCTTG-3'	1226
Sequencing fragment 1	5'-AAGCCCTTCACGATTTGG-3'	5'-TACTGCATCTTTACCCGC-3'	925

Sequencing fragment 2	5'-CGTTTTGCTACTCCTGAAGC-3'	5'-GTCATAACTTCTTTCACCACG-3'	994
Production of blunt end PCR products	5'-CACCATGGAAGTAAAGGC-3'	5'-TTATTTATCTTCTTTCTC-3'	1330

3.1. Passive immunisation

3.1.2. Lyophilisation of hyperimmune egg yolk: effect on antibody titer and protection of broilers against *Campylobacter* colonization

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ABSTRACT

Oral administration of antibodies is a promising strategy against various infectious diseases. Previously, it was demonstrated that passive immunisation by providing hyperimmune egg yolk through the feed reduces *Campylobacter jejuni* (*C. jejuni*) colonization in broilers. Campylobacteriosis is the most commonly reported bacterial foodborne zoonosis worldwide and poultry products are the number one origin of these bacteria for human infection. To date, no effective control measures exist to limit *Campylobacter* colonization in the chicken's intestinal tract. Here, the effect of lyophilisation of hyperimmune egg yolk on protection of broilers against *C. jejuni* was investigated. During an *in vivo* trial, broiler chickens were prophylactically given feed with lyophilized hyperimmune or non-immunized egg yolk powder starting from day 1 after hatch. At day 11, broilers were inoculated with *C. jejuni* according to a seeder model. Five days later, all broilers were euthanized and cecal content was examined for *C. jejuni* colonization. No decrease in *C. jejuni* colonization was found. The freeze-drying resulted in a 16-fold decrease of the antibody titer in the yolk powder compared to the fresh yolks, presumably caused by structural changes in the antibodies. In conclusion, applying freeze-dried hyperimmune egg yolk failed to protect broilers against *C. jejuni* colonization, possibly because lyophilisation affected the antibodies' functionality.

Key words: *Campylobacter*, broiler, passive immunisation, lyophilisation, bacterin

INTRODUCTION

Oral administration of antibodies is a promising strategy for controlling enteric bacterial and viral infections in humans and animals, such as *Salmonella enterica*, *Escherichia coli*, *Campylobacter jejuni* (*C. jejuni*), *Clostridium perfringens*, rotaviruses and coronavirus (Mine and Kovacs-Nolan, 2002; Schade et al., 2005; Chalghoumi et al., 2009a; Yegani and Korver, 2010; Gadde et al., 2015; Hedegaard and Heegaard, 2016). A cost-efficient method to produce such antibodies is through hyperimmunisation of chickens and collecting the egg yolks, containing high amounts of antibodies (Bizanov, 2017).

We recently demonstrated that passive immunisation using bacterin-induced antibodies was able to reduce *C. jejuni* infection in broilers (Vandeputte et al., 2019a). *Campylobacter* infection is the most commonly reported bacterial foodborne zoonosis in the European Union since 2005 (EFSA, 2017) and is mainly derived from poultry products. In most cases, clinical symptoms such as fever and diarrhoea are self-limiting. However, complications may occur, such as reactive arthritis (Hannu et al., 2002) and Guillain-Barré syndrome (Nachamkin, 2002). Unfortunately, no effective measures to control *Campylobacter* infection in poultry exist to date (Hermans et al., 2011b). In our previous study (Vandeputte et al., 2019a), vaccination of layer hens resulted in a high and prolonged immune response, observed as the presence of high amounts of anti-*Campylobacter* IgY in the egg yolk. This hyperimmune yolk was administered to the feed of the broilers at a concentration of 5%, resulting in a decrease of infected birds from 78% to 15%. However, under field conditions, egg yolk as such cannot be implemented and therefore an alternative administration method for these antibodies should be developed.

Lyophilisation of egg yolk results in an easy-to-mix egg yolk powder (EYP) with an extended shelf life. Moreover, the yolk is considered to form a protective matrix for the antibodies

during their passage through the gastrointestinal tract (Schade et al., 2005) and to contain antimicrobial components (Kassaify and Mine, 2004a,b), so it would be advantageous to preserve these beneficial characteristics. Here, we investigated if we could obtain a colonization reduction similar to our previous results using hyperimmune EYP.

MATERIAL AND METHODS

Experimental animals

Commercial Ross 308 broiler chickens of both sexes were purchased at a local hatchery (Vervaeke-Belavi, Tielt, Belgium). The animals were provided with a commercial feed and water ad libitum. Husbandry, experimental procedures, euthanasia methods and bio-safety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium and in accordance with the relevant guidelines and regulations (EC2016/28). Birds were proved to be free of *Campylobacter* by examination of mixed fecal samples using standard methods as described by Hermans et al. (2011a).

Bacterial strains and culture conditions

For the experimental infection, *C. jejuni* reference strain KC40 from poultry origin was used, which colonizes chickens to a high level (Van Deun et al., 2008). 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 for 17 h under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2). *C. jejuni* bacteria in the broth were enumerated by plating tenfold dilutions in Hank's Balanced Salt Solution (HBSS;

GIBCO-BRL, Invitrogen, Carlsbad, CA) 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.

Preparation of hyperimmune egg yolk powder

Hyperimmune egg yolks against a bacterin mix of thirteen genetically diverse *C. jejuni* and *C. coli* strains and egg yolks from sham-immunized hens were previously produced by Vandeputte et al. (2019a) and further processed at the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO, Melle, Belgium). Before lyophilization, the yolks were stored at 4°C. The yolks were frozen at -50°C during 2 h and then lyophilized (sublimation: 0.16 mbar, 20 h -50°C to 20°C, 20 h at 20°C; desorption: 0.025 mbar, 2 h 20°C to 30°C, 3 h at 30°C; condenser temperature: -90°C) in an Epsilon 2-10 D LSC freeze-drier (Martin-Christ, Osterode am Harz, Germany). The hyperimmune and non-immunized EYP (resp. HEYP and NEYP) were stored at 4°C until further processing.

Prophylactic efficacy of in-feed supplementation of bacterin derived HEYP on *C. jejuni* cecal colonization in broilers

Fifty-four day-of-hatch *Campylobacter* free broilers were raised in two randomly assigned treatment groups (n = 27/group) and housed in separate isolation units. From the day of hatch until the end of the experiment, the chicks were provided with feed containing 2.5% (wt/wt) HEYP (group 1) or NEYP (group 2), mixed manually through the feed. This concentration approaches the 5% egg yolk content used by Vandeputte et al. (2019a). Groups administered fresh hyperimmune and non-immunized yolk were not repeated for ethical reasons, to reduce

the number of animals. 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 10 days of age, the chicks of each group were randomly assigned to three subgroups (n = 9/subgroup) and housed in separate isolation units. At 11 days of age, three seeder chicks of each subgroup were randomly selected and orally inoculated with approximately 1 x 10^5 cfu of *C. jejuni* strain KC40, quantified by plating as described above. The birds that were not inoculated are referred to as sentinels. At day 16, all animals were euthanized by injection of an overdose (100 mg/kg) sodium pentobarbital (Kela, Hoogstraten, Belgium) in the wing vein and the cecal content was collected for *C. jejuni* enumeration (as described below).

Cecal *C. jejuni* enumeration

Cecal *C. jejuni* enumeration was performed by qPCR as described by Vandeputte et al. (2019b), originally adapted from Lund et al. (2004) and Botteldoorn et al. (2008). Briefly, DNA-extraction was performed using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions, with the single adaptation that the DNA was eluted in 100 µL instead of 200 µL ATE buffer. The DNA was stored at -20°C until further analysis.

Per qPCR reaction, 12.5 µL IQ™ Supermix (Bio-rad, Temse, Belgium), 0.25 µL of each primer (forward primer Camp2F: 5' CACGTGCTACAATGGCATAT 3', reverse primer Camp2R: 5' GGCTTCATGCTCTCGAGTT 3'), 0.25 µL probe (Camp2P: 5' 6FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1 3'), 6.75 µL HPLC-water and 5 µL sample DNA was mixed until a total volume of 25 µL. Primers and probe were purchased at Integrated DNA Technologies e IDT (Leuven, Belgium). After centrifuging for 1 min at 1500

tpm, the following qPCR program was run: 2 min at 50°C, 10 min at 95°C and 50 cycles of 15 sec at 95°C and 60 sec at 60°C (CFX96 Real-Time PCR Detection System, Bio-rad). The number of *C. jejuni* in the cecal content was expressed as genomic equivalents (ge)/g cecal content.

Determination of egg yolk IgY titers

Campylobacter-specific IgY titers in egg yolks were determined before and after lyophilisation, as previously described by Hermans et al. (2014) with minor changes to the protocol. Egg yolks were diluted 1/5 (vol/vol) in HBSS, mixed thoroughly and incubated overnight at 4°C. Lyophilized egg yolks were first diluted 1/2 (wt/vol) in HBSS, since half of the egg yolk consists of water (Bizanov, 2017). The supernatant, containing the water-soluble fraction of the egg yolk, was collected for IgY quantification using ELISA. To determine egg yolk IgY titers, 96 well flat bottom plates (Nunc MaxiSorp, Nalge Nunc Int., Rochester, NY, USA) were coated (24 h, 4°C) with 10^6 cfu (before killing) bacterin diluted in 50 µL coating buffer (2.16 g $Na_2CO_3.10H_2O$, 1.935 g $NaHCO_3$ in 500 mL H_2O). After washing (3x HBSS, 1x washing buffer: 0.1% Tween-20 in PBS), the wells were blocked (1 h, room temperature) with 100 µL blocking buffer (1% BSA in washing buffer). Next, 100 µL of a 1/2 dilution series of the supernatant of the mixed egg yolks was incubated during 60 min at room temperature. Plates were washed as described above and incubated with 100 µL 1/10.000 horseradish peroxidase (HRP)-labelled anti-chicken IgY (Sigma Aldrich) in washing buffer during 90 min at room temperature. After washing as described above, the plates were incubated with 50 µL 3,3',5,5'-tetramethyl benzidine (TMB) substrate (Sigma Aldrich) for 10 min at room temperature in the dark. Next, 50 µL 0.5M H_2SO_4 was added to each well and the absorbance at 450 nm (OD_{450}) was measured using an automated spectrophotometer

(Pharmacia LKB Ultrospec III, Gemini BV, Apeldoorn, Nederland). IgY titers from yolks of immunized hens were reported as the highest dilution where the OD_{450} was greater than the OD_{450} + three standard deviations of wells containing yolk originating from sham vaccinated birds.

Statistical analysis

Data of the *in vivo* trial were analyzed using R 3.3.1. Before statistical analysis, *C. jejuni* numbers were transformed to $\log_{10}$ numbers. The colonization data were analyzed using a hurdle model, a class of models that assumes that the data are generated by two processes. First, the event that an individual is colonized (i.e. returning a non-zero count) follows a Bernoulli distribution. Given colonization, its intensity or load is a random variable following a discrete or continuous distribution; in this case, a gamma distribution was assumed. The model was used to estimate the probability of *C. jejuni* colonization and the mean *C. jejuni* numbers in the cecal content of colonized birds for each treatment level. Next, the pairwise differences between those, and the proportion of the respective posterior distributions that had the same sign as the mean were calculated. If working in a null-hypothesis significance testing framework, this can be interpreted as a one-sided test, estimating the probability that the true difference between treatments is zero or greater (if negative) or smaller (if positive), and thus the level of confidence that the null hypothesis can be rejected. For a detailed description of the implementation in R, reference is made to Vandeputte et al. (2019a).

RESULTS

Protective effect of prophylactic passive immunisation of broilers with bacterin derived HEYP against cecal *C. jejuni* colonization

C. jejuni numbers per gram cecal content after euthanasia of the chickens, prophylactically fed HEYP or NEYP, are given in Table 1 and Figure 1. No significant reduction of the number of *C. jejuni* colonized broilers was observed in HEYP-treated subgroups compared to the control subgroups (resp. 21/27, 24/27; $P > 0.05$). In each subgroup of both treatments, all seeders were colonized. The difference in the number of colonized birds between the two groups was a consequence of the variation in *C. jejuni* transmission to the sentinel birds. Yet, this reduction was not significant (resp. 12/18, 15/18; $P > 0.05$). No significant decrease of the mean *C. jejuni* numbers in birds positive for colonization between the HEYP-treated and control groups was found [resp. 4.87, 5.11 $\log_{10}$ (ge/g cecal content); $P > 0.05$], as well as for seeders separately [resp. 5.79, 6.26 $\log_{10}$ (ge/g cecal content); $P > 0.05$] or sentinels separately [resp. 4.18, 4.41 $\log_{10}$ (ge/g cecal content); $P > 0.05$].

Effect of hyperimmune egg yolk lyophilisation on antibody titers

The bacterin-induced *Campylobacter*-specific IgY titer in the egg yolks before lyophilisation was 1:65,536, as determined by ELISA. After lyophilisation, the antibody titer was reduced to 1:4,096.

Table 1. Number of positive birds and mean cecal *C. jejuni* numbers of colonized broilers receiving standard feed supplemented with 2.5% (wt/wt) egg yolk powder from either bacterin-immunized or sham-immunized (control) layers, from day 1 until day 16 (the day of euthanasia). At 11 days of age, 3 seeder birds per group were inoculated with approximately 10^5 cfu *C. jejuni* KC40. A random effect was included in the statistical model at the subgroup (pen) level to account for clustering.

	Number of positive birds		**Mean *C. jejuni* numbers of positive birds (log_{10}(cfu/g cecal content)) (*Standard deviation*)**	
	Bacterin	**Control**	**Bacterin**	**Control**
All birds				
Group 1	8/9	6/9	5.05 (*1.58*)	5.84 (*1.33*)
Group 2	6/9	9/9	3.82 (*1.44*)	5.14 (*1.19)*
Group 3	7/9	9/9	5.57 (*1.93*)	4.59 (*1.03*)
Treatment	**21/27[a]**	**24/27[a]**	**4.87[a] (*1.74*)**	**5.11[a] (*1.60*)**
Seeders				
Group 1	3/3	3/3	5.71 (*2.31*)	6.65 (*1.19*)
Group 2	3/3	3/3	4.53 (*1.83*)	6.33 (*0.90*)
Group 3	3/3	3/3	7.13 (*0.58*)	5.82 (*1.02*)
Treatment	**9/9[a]**	**9/9[a]**	**5.79[a] (*1.88*)**	**6.26[a] (*0.97*)**
Sentinels				
Group 1	5/6	3/6	4.66 (*1.08*)	5.03 (*1.03*)
Group 2	3/6	6/6	2.32 (*0.56*)	4.55 (*2.00*)
Group 3	4/6	6/6	4.41 (*1.73*)	3.97 (*1.21*)
Treatment	**12/18[a]**	**15/18[a]**	**4.18[a] (*1.32*)**	**4.41[a] (*1.51*)**

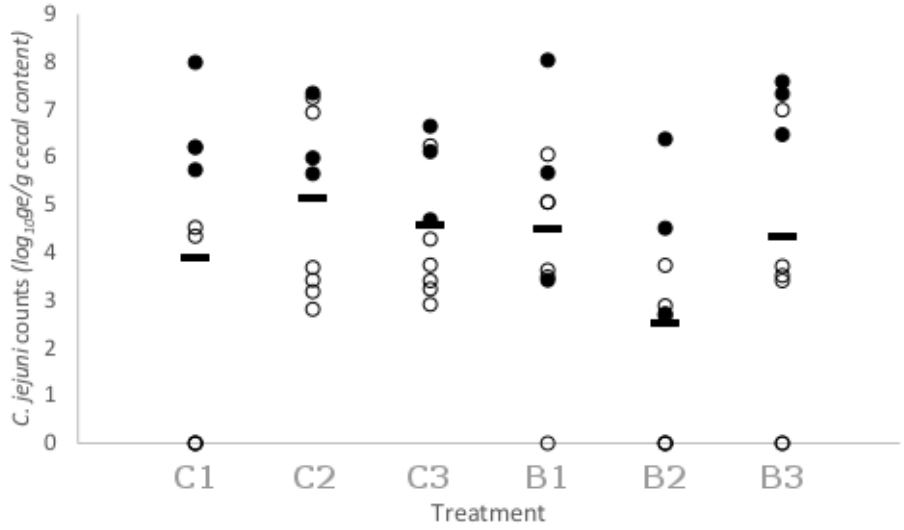

Figure 1. Individual and mean (—) cecal *C. jejuni* numbers of colonized broiler seeders (●) and sentinels (○) after prophylactic treatment with lyophilized hyperimmune egg yolk. The birds received standard feed supplemented with 2.5% (wt/wt) lyophilized egg yolk from either sham-immunized (C1, C2, C3) or bacterin-immunized (B1, B2, B3) layers, from day 1 until day 16 (the day of euthanasia). At 11 days of age, seeder birds were inoculated with approximately 10^5 cfu *C. jejuni* KC40. Values are represented as $\log_{10}$ ge/g cecal content.

DISCUSSION

In the present study, no protection against *C. jejuni* colonization was detected in chickens fed HEYP compared to control animals. This is in contrast to the results of Hermans et al. (2014) and Vandeputte et al. (2019a), the latter applying the same vaccines and procedures as used in this study, but using fresh yolks. A possible explanation is that lyophilisation resulted in a 16-fold decline of the *Campylobacter*-specific antibody titer in the yolk, as determined by ELISA. Previously, Shimizu et al. (1988) found no effect of lyophilisation on IgY activity,

while others showed that freeze-drying resulted in some loss of antigen-binding activity of IgY (Sunwoo et al., 2002). Lyophilization of proteins induces freezing and dehydration stresses, which may result in protein structural changes or even unfolding (Emami et al., 2018). Therefore, to protect the proteins against these stresses, these authors recommend adding cryo- and lyoprotectants.

As noted by Chalghoumi et al. (2009b) and Paul et al. (2014), the initial antibody dose, when orally administered, should be sufficiently high to ensure that an adequate amount of functional antibodies survives the gastrointestinal passage to establish protection against bacterial colonization. It is possible that the remaining titer after lyophilisation (>1:4.096) was insufficient compared to the titers supplemented by Vandeputte et al. (2019a) using fresh yolks (>1:65.536).

The need to protect the antibodies during the passage through the gastrointestinal tract, against for example the acidic stomach environment and proteases, has been emphasized before (Schade et al., 2005, Chalghoumi et al., 2009a). Egg yolk is considered to form a protective matrix for the antibodies against degradation and functionality loss during this passage (Schade et al., 2005), but this protection might be affected during the freeze-drying process. Other methods can be applied to increase stability of the antibodies, such as micro-encapsulation, however this would increase the production complexity and cost (Chalghoumi et al., 2009a).

In conclusion, it was demonstrated that administering freeze-dried hyperimmune yolk did not protect broilers against *C. jejuni* colonization, in contrast to a previous study using fresh yolks. This is correlated with decreased antigen binding after lyophilisation and may be further compromised by degradation during gastro-intestinal passage. Further research will be needed to develop an industrially applicable method to administer these antibodies.

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3.2. Active immunisation

In ovo vaccination of broilers against *Campylobacter jejuni* using a bacterin and subunit vaccine

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ABSTRACT

Campylobacter jejuni (*C. jejuni*) and *C. coli* originating from poultry meat have been the most important causes of foodborne bacterial gastroenteritis in the European Union since 2005. In-feed application of maternal antibodies from vaccinated hens was shown to confer protection of broilers against *Campylobacter* infection. Here, it was investigated if these vaccines can be used to protect broilers against *Campylobacter* infection after *in ovo* vaccination. Embryos were immunized *in ovo* at day 18 with a bacterin or a subunit vaccine and at 19-days post hatch, these birds were inoculated with *C. jejuni* according to a seeder model. Quantification of *C. jejuni* in the broilers cecal content showed that the *in ovo* vaccinated birds were not protected against *C. jejuni* infection. Quantification of blood anti-*Campylobacter* antibody titers did not show any induction of *Campylobacter*-specific serological response in the vaccinated birds, which may explain the lack of protection in the vaccinated chicks.

Key words: *Campylobacter*, broiler, *in ovo* vaccination, bacterin, subunit vaccine

INTRODUCTION

Campylobacter has been the most commonly reported cause of foodborne human bacterial gastroenteritis in the European Union since 2005 and broiler meat is considered to be its most important source (EFSA, 2017). Except for biosecurity measures, few options are available to control *Campylobacter* infection in poultry (Hermans et al., 2011). Although immunisation is a promising method (Hermans et al., 2011), no vaccine has been commercialized yet. Earlier, a bacterin mix of thirteen heterologous *Campylobacter* strains and a subunit vaccine of six conserved, immunodominant *Campylobacter* antigens, induced a high and prolonged immune response in layer hens and reduced *Campylobacter jejuni* (*C. jejuni*) colonization of broilers when used for passive immunisation (Vandeputte et al., 2019). This passive immunisation consisted of orally supplementing broiler feed with high levels of anti-*Campylobacter* specific antibodies, by adding egg yolks from immunized hens. However, the use of fresh yolks would be impractical to apply in the industry and other administration methods should be investigated. These vaccines may be candidates to vaccinate broiler chicks against *Campylobacter* infection through another administration route.

Although the chicken immune system is only fully developed at several weeks after hatching, the 18-day-old embryo is capable of reacting to an administered antigen (Avakian et al., 2007). *In ovo* vaccination is a safe and user-friendly method (Peebles, 2018). Many diseases have been researched, such as Marek's disease (Sharma and Burmester, 1982, Peebles et al., 2016), infectious bursal disease (Giambrone et al., 2001, Moura et al., 2007), Newcastle disease (Stone et al., 1997, Palya et al., 2011), avian influenza (Stone et al., 1997, Mesonero et al., 2011), coccidiosis (Sokale et al., 2017) and *Mycoplasma gallisepticum* (Elliot et al., 2017) and *in ovo* vaccination has already been approved for Marek's disease, infectious bursal disease and Newcastle disease (Peebles, 2018). Noor et al. (1995) were the first to demonstrate a humoral immune response in the serum, intestine, bile and spleen of broilers

against *Campylobacter* after *in ovo* vaccination with a heat-killed *C. jejuni* isolate. Unfortunately, the chickens were not challenged and protection was not investigated. It was only until recently that others started researching the potential protection against *Campylobacter* sp. by *in ovo* vaccination (Kobierecka et al., 2016). Radomska et al. (2016) used a recombinant flagellin-based subunit vaccine with intrinsic adjuvant activity and obtained serum IgY and IgM induction, but no mucosal immune response or protection. In another study, combining the hybrid protein rCjaAD (CjaA presenting CjaD epitopes) with Gram-positive enhancer matrix (GEM) particles or liposomes resulted in some protection against a heterologous *C. jejuni* strain (Kobierecka et al., 2016). At the same time, Godlewska et al. (2016) used outer membrane vesicles (OMVs) which resulted in a maximum reduction of 1 $\log_{10}$ of cecal *Campylobacter* colonization.

In this study, it was investigated if *in ovo* vaccination with the bacterin or subunit vaccine (Vandeputte et al., 2019), containing an adjuvant used successfully before for *in ovo* vaccination (Lillehoj et al., 2017), could protect chickens against *Campylobacter* infection.

MATERIALS AND METHODS

Experimental animals

Commercial Ross 308 broiler chicken eggs were purchased at 18 days of incubation at a local hatchery (Vervaeke-Belavi, Tielt, Belgium) and incubated in a MARU Hatcher & Brooder 380 (Maru HB380, Rcom, Wichita, Kansas, United States) at 37°C and 65% humidity. After hatching, the animals were provided with a commercial feed and water *ad libitum*. Husbandry, experimental procedures, euthanasia methods and biosafety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University, Ghent, Belgium (Ethical Committee number: 2017/104).

Birds were proved to be free of *Campylobacter* by examination of mixed fecal samples by qPCR (as described below).

Bacterial strains and culture conditions

For the experimental infection in the *in vivo* trial, *C. jejuni* reference strain KC40 from poultry origin was used, which colonizes chickens to a high level (Van Deun et al., 2008). Bacteria were routinely obtained from a frozen stock (-70°C) and cultured on modified charcoal cefoperazone deoxycholate agar (mCCDA, CM0739; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with CCDA selective supplement (SR0155E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid) and in Nutrient Broth No.2 (NB2, CM0067; Oxoid) supplemented with Modified Preston *Campylobacter*-selective supplement (SR0204E; Oxoid) and *Campylobacter*-specific growth supplement (SR0232E; Oxoid), at 42°C under microaerobic conditions (5% O_2, 5% CO_2, 5% H_2, 85% N_2).

Bacterin and subunit vaccine preparation

The bacterin and subunit vaccine were developed as described by Vandeputte et al. (2019). Briefly, 13 genetically heterogeneous *Campylobacter* strains, including the *C. jejuni* KC40 reference strain, killed by formaldehyde, were included in the bacterin. Each bacterin dose (volume of 50 µL) consisted of 6.3 $\log_{10}$ colony forming units (cfu) of each *Campylobacter* strain (n=13), resulting in a total of 7.4 $\log_{10}$ cfu inactivated *Campylobacter*/bacterin dose. The subunit vaccine consisted of six immunodominant *Campylobacter* antigens (AtpA, CheV, EfTu, GroEL, LivJ and Tig), produced recombinantly using Gateway® Technology (Invitrogen, Carlsbad, CA). For this, 4.75 µg of each antigen (i.e. 28.5 µg in total) was supplemented with Hank's Balanced Salt Solution (HBSS; GIBCO-BRL, Invitrogen,

Carlsbad, CA) until a volume of 50 µL/vaccine dose. For sham immunisation, 50 µL HBSS was used (negative control). Each immunisation dose consisted of 100 µL of a 1:1 mixture of the inoculum with ESSAI IMS 1505101 OVO 1 adjuvant (SEPPIC, Paris, France). Sterility of the vaccines was checked by plating on Columbia agar with 5% sheep blood (PB5039A; Oxoid) and evaluation of the plates for bacterial growth after 72 h incubation at 37°C under aerobic conditions.

In ovo vaccination of broiler chicken embryos

At 18 days of incubation, eighty two eggs were randomly assigned to three groups (control, bacterin and subunit vaccine), labeled and immersed in 0.5% sodium hypochlorite to disinfect the egg shell. The egg was punctured using a needle, which was disinfected with 70% isopropyl alcohol for 2 minutes. Via this puncture, 100 µL vaccine was injected into the amniotic cavity. Vaccinated eggs were labeled immediately and incubated as described above. At 21 days of incubation, 98% of the eggs hatched and no adverse effects of the vaccination were observed in these animals. Mortalities after *in ovo* vaccination were not encountered.

Efficacy of *in ovo* vaccination on cecal colonization with *C. jejuni* in broilers

After hatching, the broiler chicks were labeled and housed together, as described above. At day 18 after hatching, 27 chickens of each vaccine group (control, bacterin and subunit vaccine) were randomly selected and housed in 3 subgroups in separate isolation units (n = 9/subgroup). Blood samples from the wing vein were collected from each of these chickens. At 19 days of age, three seeder chicks of each subgroup were randomly selected and orally inoculated with approximately 1 x 10^7 cfu of *C. jejuni* strain KC40. The concentration of the

inoculum was confirmed by OD_{600} measurement and plating of serial dilutions before and after inoculation. The birds that were not inoculated are referred to as contact animals or sentinels. At day 24, all animals were euthanized by injection of an overdose (100 mg/kg body weight) sodium pentobarbital (Kela, Hoogstraten) in the wing vein. Blood samples were collected from the *vena jugularis* and the cecal content was collected for *C. jejuni* enumeration (as described below).

Fecal and cecal *C. jejuni* enumeration

DNA-extraction was performed using the QIAamp® Fast DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions, with the single adaptation that the DNA was eluted in 100 µL instead of 200 µL ATE buffer. The DNA was stored at -20°C until further analysis.

Quantitative PCR (qPCR) analysis was performed as described before (Lund et al., 2004; Botteldoorn et al., 2008). Briefly, per qPCR reaction, 12.5 µL IQ™ Supermix (Bio-rad, Temse, Belgium), 0.25 µL of each primer (forward primer Camp2F: 5' CACGTGCTACAATGGCATAT 3', reverse primer Camp2R: 5' GGCTTCATGCTCTCGAGTT 3'), 0.25 µL probe (Camp2P: 5' 6FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1 3'), 6.75 µL HPLC-water and 5 µL sample DNA was mixed until a total volume of 25 µL. Primers and probe were purchased at Integrated DNA Technologies (Leuven, Belgium). After centrifuging for 1 min at 1500 rpm, the following qPCR program was run: 2 min at 50°C, 10 min at 95°C and 50 cycles of 15 sec at 95°C and 60 sec at 60°C (CFX96 Real-Time PCR Detection System, Bio-rad).

Antibody response to bacterin and subunit vaccine after *in ovo* vaccination

Blood samples were incubated at room temperature for 30 min and overnight at 4°C. The samples were then centrifuged (10 min, 1000 g, 4°C) and the supernatant was stored at -20°C until determination of anti-*Campylobacter* antibody titers.

Campylobacter-specific IgM, IgY and IgA titers were determined by ELISA based on the protocol described by Hermans et al. (2014). The plates (96 well flat bottom plates, Nunc MaxiSorp, Nalge Nunc Int., Rochester, NY, USA) were coated (24 h, 4°C) with 10^6 cfu bacterin diluted in 50 µL coating buffer (15.1 mM $Na_2CO_3.10H_2O$, 46.2 mM $NaHCO_3$ in H_2O). After washing [3x HBSS, 1x washing buffer: 0,1% Tween-20 (VWR International, France) in PBS], the wells were blocked (1 h, 21°C) with 100 µL blocking buffer [1% BSA in washing buffer]. Next, 100 µL of a 1/200 dilution of the serum sample was incubated during 60 min at room temperature. Plates were washed as described above and incubated with 100 µL secondary antibody [1/5,000 goat anti-chicken IgM secondary antibody (horseradish peroxidase, HRP) (LifeSpanBioSiences, Huissen, The Netherlands); 1/10,000 HRP-labelled anti-chicken IgY (Sigma Aldrich); 1/5,000 goat anti-chicken IgA secondary antibody (HRP) (LifeSpanBioSiences)] in washing buffer during 90 min at room temperature. After washing as described above, the plates were incubated with 50 µL 3,3',5,5'-tetramethyl benzidine (TMB) substrate (Sigma Aldrich) for 10 min at room temperature in the dark. Next, 50 µL 0.5 M H_2SO_4 was added to each well and the absorbance at 450 nm (OD_{450}) was measured using an automated spectrophotometer (Pharmacia LKB Ultrospec III, Gemini BV, Apeldoorn, Nederland). Blood originating from sham-vaccinated layer hens, confirmed to be *Campylobacter*-free, was included as a control for the absence of maternal antibodies (MAB) (Vandeputte et al., 2019).

Statistical analysis

Data of the *in vivo* trial were analyzed using R 3.3.1. The significance level α for all analyses was set at 0.05. *C. jejuni* ge were transformed to $\log_{10}$ ge and binary data before statistical analysis. First, to determine whether vaccination induced an immune response, a two-way ANOVA comparing the blood antibody titers before inoculation (day 1 and day 18) among treatment groups (control, bacterin and subunit vaccine) was performed. Second, the proportion infected of all birds (binary data) and $\log_{10}$ ge of *Campylobacter*-positive birds were compared using a hurdle model (package glmmADMB in R) (Vandeputte et al., 2019). Treatment group, antibody titer before inoculation and bird type (seeder, sentinel) were included as fixed effects and a random effect at the pen level to account for clustering. Third, the influence of infection on the difference in blood antibody titers before (day 18) and after (day 24) inoculation was analysed by a two-way ANOVA. Again treatment group, antibody titer before inoculation and bird type were included as fixed effects and a random effect at the pen level to account for clustering.

RESULTS

Protective effect of *in ovo* vaccination of broiler embryos with the bacterin or subunit vaccine against *C. jejuni*

C. jejuni genomic equivalents (ge) per gram cecal content after euthanasia of the chickens are summarized in Figure 1. No reduction of the number of *C. jejuni* colonized broilers was observed in bacterin- or subunit vaccine-immunized groups compared to the sham-vaccinated groups (resp. 21/27, 21/27, 17/27; $P > 0.05$). In infected birds, average cecal *C. jejuni* numbers were similar for all groups (resp. 6.07, 5.54, 5.80 $\log_{10}$ ge/g cecal content; $P > 0.05$).

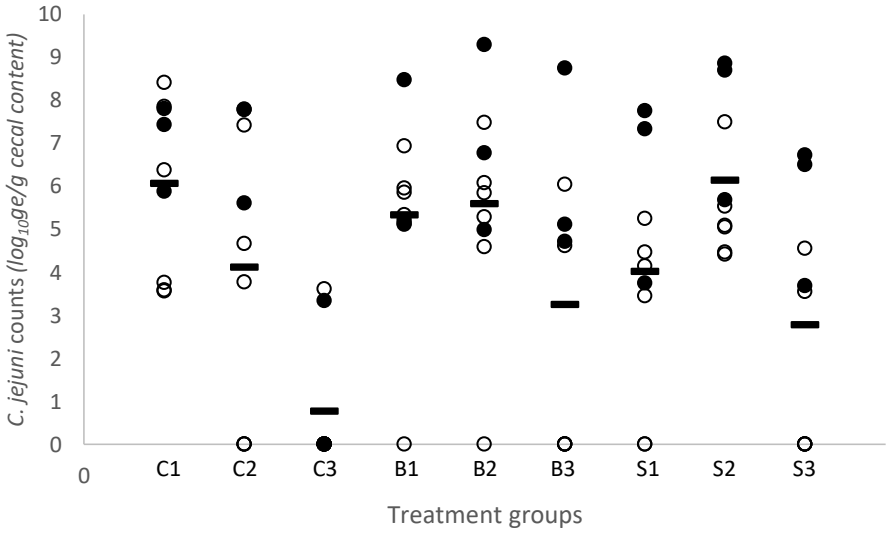

Figure 1. Individual and mean (─) cecal *C. jejuni* numbers of broiler seeders (●) and sentinels (○) *in ovo*-vaccinated with either a sham (C1, C2, C3), bacterin (B1, B2, B3) or subunit vaccine (S1, S2, S3). At 19 days of age, seeder birds were inoculated with approximately 10^7 cfu *C. jejuni* KC40. Birds were euthanized at day 24 and *C. jejuni* numbers in their cecal content were determined. Values are represented as $\log_{10}$ ge/g cecal content. Values below the detection limit are represented on the figure at zero.

Serological response after *in ovo* vaccination of broiler embryos with the bacterin or subunit vaccine against *C. jejuni*

No induction of IgM, IgY or IgA titers was found at day 18 post hatch after vaccination with the bacterin or subunit vaccine compared to the sham-vaccinated birds ($P > 0.05$) (Figure 2a). Sham-vaccinated broilers did not have elevated anti-*Campylobacter* titers compared to the MAB-negative group ($P > 0.05$). Also, no increase in IgM, IgY or IgA titers was found in infected birds five days after inoculation ($P > 0.05$) (Figure 2b).

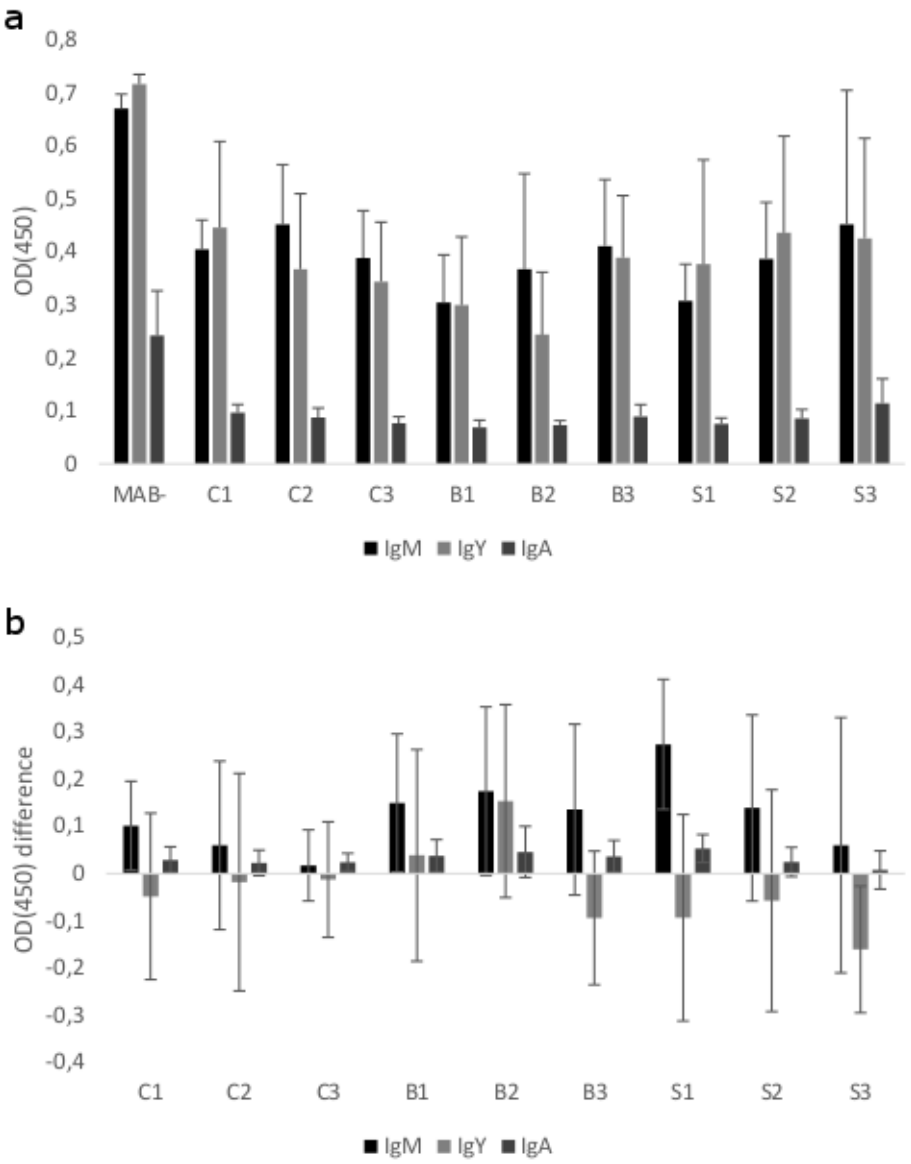

Figure 2. (a) Mean blood IgM, IgY and IgA OD_{450} values at day 18 post hatching of broilers *in ovo*-vaccinated with either a sham (C1, C2, C3), bacterin (B1, B2, B3) or subunit vaccine (S1, S2, S3). As a control for the absence of maternal antibodies (MAB-), blood originating from sham-vaccinated layer hens was included (Vandeputte et al., 2019). At 19 days of age, seeder birds were inoculated with approximately 10^7 cfu *C. jejuni* KC40. (b) Mean of blood antibody OD_{450} value increase after inoculation (difference between day 18 and day 24).

DISCUSSION

In this study, the efficacy of *in ovo* vaccination of broiler embryos against *C. jejuni* using a bacterin or subunit vaccine was investigated. Previously, it was shown that these vaccines induced a high and prolonged immune response in layer hens and were able to reduce *C. jejuni* infection in broilers by passive immunisation (Vandeputte et al., 2019). However, no measurable serological response was induced in our experiment and consequently, the vaccinated broilers were not protected against *C. jejuni* infection.

The discrepancy between the immune responses may come from the difference in stage of immune development or genetic background between the 20-week-old layers and 18-day-old broiler embryos (Avakian et al., 2007; Psifidi et al., 2016). In addition, the layers in the passive immunisation study were boosted thrice, whereas the broilers in this study did not receive any subsequent vaccination because the scope was to investigate if *in ovo* vaccination without labor-intensive boosters could be sufficient. Maternal antibodies present in the embryo may interfere with *in ovo* vaccination (Negash et al., 2004). During this experiment, sham-vaccinated broilers did not have elevated anti-*Campylobacter* titers compared to the MAB-negative group, indicating the absence of maternal immunity. Another factor that might influence the immune response, is the adjuvant used. The vaccines in the passive immunisation study were composed with Freund's adjuvant. For this study, a less toxic adjuvant (Sivakumar et al., 2011) was selected, which was successfully used before for *in ovo* vaccination of broilers (Lillehoj et al., 2017).

Kobierecka et al. (2016) also concluded that vaccination results can vary depending on the administration strategy. The authors failed to protect chickens against *C. jejuni* using a subunit vaccine by oral or subcutaneous delivery, but obtained a 1-2 $\log_{10}$ reduction of *C. jejuni* numbers in the cecal content by *in ovo* vaccination when using liposomes as a carrier.

Another example is the research of Annamalai et al. (2013), where subcutaneous vaccination with outer membrane proteins significantly reduced *Campylobacter* colonization, but oral delivery of the same vaccine did not. Together, these studies, when administering an inoculum via different routes, demonstrate the importance of the immunisation strategy.

To improve our vaccination strategy, a vector or carrier might be added to the subunit vaccine. A commonly used vector for *in ovo* vaccination is the herpesvirus of turkeys (Gimeno, 2008), on which antigens of multiple pathogens can be combined. Alternatively, the antigens can be carried on GEM particles or liposomes (Kobierecka et al., 2016) or OMVs (Godlewska et al., 2016). Also, a booster vaccination can be administered after hatching to increase the immune response of the chickens. In future studies, also the mucosal immune response can be measured. In conclusion, more research will be needed to develop an effective and applicable *in ovo* vaccine.

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CHAPTER 4: GENERAL DISCUSSION

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The need to control *Campylobacter* in poultry

Campylobacter is the cause of the most important bacterial zoonosis since 2005, affecting 246,158 people in the EU in 2017, which results in an estimated annual cost of €2.4 billion (EFSA, 2018). Since poultry form the main source for human infection (Berndtson et al., 1992; Friedman et al., 2004; EFSA, 2018), measures to control this bacterium in poultry should be developed. Antibiotics should not be used due to emerging resistance (Dibner and Richards, 2005; Zhu et al., 2006) and control measures are currently limited to hygiene and biosafety measures (Hermans et al., 2011a). The high number of human campylobacteriosis cases indicates that the current implementation of these measures is not sufficient.

Immunisation could be an effective method to limit *Campylobacter* in poultry (de Zoete et al., 2007). The aim of this thesis was to investigate the efficacy and applicability of two such methods: passive immunisation using hyperimmune egg yolks and active immunisation by *in ovo* vaccination.

Efficacy of passive immunisation as a measure to control *Campylobacter* colonization

The interesting finding that young chicks are protected against *Campylobacter* colonization until an age of two to three weeks is probably due to the protection by maternal antibodies, passed along through the egg yolk (Sahin et al., 2003), although the further development of immunity (Friedman et al., 2003; Bar-Shira and Friedman, 2006) and a shift in the microbial community (van Der Wielen et al., 2000; Ngunjiri et al., 2019; Richards et al., 2019) may also play a role. This brings new possibilities to combat *Campylobacter*: can these antibodies

be used to prevent infection of older birds, or to reduce *Campylobacter* titers in the intestinal tract before slaughter?

Tsubokura et al. (1997) were the first to demonstrate the prophylactic and therapeutic effect of anti-*Campylobacter* antibody preparations from chicken eggs and bovine milk. More recently, Hermans et al. (2014) have shown that it is indeed possible to reduce *Campylobacter* titers in infected birds and transmission to contact animals by administering hyperimmune egg yolks through the feed. These antibodies were raised against a cell lysate or the hydrophobic protein fraction of a single isolate, the *C. jejuni* KC40 strain. In the field, many heterologous strains are present, which may infect the chicken and may succeed each other as the dominating strain (Skånseng et al., 2007; Ridley et al., 2008). Therefore, antibodies raised by vaccination should account for this wide variety.

In the first study, two novel vaccines were composed: a bacterin and a subunit vaccine. Immunisation of layer hens with one of these vaccines resulted in a prolonged antibody response, as detected in their egg yolks. Moreover, the antibodies showed *in vitro* reactivity to the heterologous strains incorporated in the bacterin and, in the case of the bacterin-induced antibodies, also to unrelated isolates. Prophylactic administration of these egg yolks through the feed of broilers resulted in a reduced percentage of colonized animals after inoculation with the *C. jejuni* KC40 strain and therapeutic administration of the bacterin yolks resulted in reduced cecal *Campylobacter* titers. In both experiments, the bacterin yolks gave better results than the subunit vaccine yolks, which can be explained by the fact that the bacterin mix probably contains more epitopes than the subunit vaccine. When combining this with the finding that the bacterin-induced antibodies have a broader cross-reactivity to unrelated strains, follow-up experiments preferably apply the bacterin treatment.

Better results were obtained with the preventive treatment. Using the bacterin yolks, this treatment resulted in a decrease from 78% to 15% of infected broilers compared to control birds. Interestingly, this is only half of the percentage of inoculated seeder birds. The seeder model was developed to mimic the field situation, where initially only one or a few birds get infected, from where the bacterium quickly spreads to the rest of the flock (Stern et al., 2001; van Gerwe et al., 2009). So far, immunisation appears to be the most promising method when using the seeder model. In the study by Hermans et al. (2014), transmission to the sentinels was completely inhibited by passive immunisation when using a whole cell *C. jejuni* KC40 lysate as a vaccine. On the contrary, transmission was not prevented when administering medium-chain fatty acids (a caproic, caprylic, capric and lauric acid mixture) via the drinking water of broilers (Hermans et al., 2012a), trans-cinnamaldehyde (Hermans et al., 2011b) or butyrate coated microbeads (Van Deun et al., 2008a) through the feed or after oral inoculation of a live *Enterococcus faecalis* strain (Robyn et al., 2013). Many researchers do not use a seeder model but challenge all birds in their experiments to investigate infection (Meunier et al., 2016b), although the former provides important information about transmission. However, this experimental setup still deviates from the field situation. In practice, the broilers are slaughtered at (at least) six weeks and the treatment may only delay the bacterium's spread through the flock. An estimation of the potential colonization reduction should be based on a trial closely mimicking field conditions, such as flock size, slaughter age and environmental risk factors for initial infection. Although our ELISA results are indicative for cross-protection against other *Campylobacter* strains, it should be investigated if an equally sufficient protection can be obtained *in vivo* against other strains compared to the *C. jejuni* KC40 strain.

Therapeutic administration of the bacterin yolks resulted in reduced cecal *Campylobacter* titers by a factor 17.78, or 1.25 log. Under experimental conditions, 1-3 $\log_{10}$ or higher

reductions after vaccination have also been obtained by several research groups (Widders et al., 1998; Wyszyńska et al., 2004; Buckley et al., 2010; Huang et al., 2010; Layton et al., 2011; Clark et al., 2012; Theoret et al., 2012; Neal-McKinney et al., 2014), although these studies did not yet result in the development of a commercially available vaccine. Often, a 3 $\log_{10}$ reduction is aimed at (Hermans, 2012), which is expected to correspond 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). Therefore, passive immunisation with egg yolks seems more promising when applied prophylactically.

Applicability of passive immunisation as a measure to control *Campylobacter* colonization

In the field, applying fresh yolks in the feed is not practical, due to the short conservation period and high viscosity, complicating mixing the yolks through the feed. Another method should be developed to administer these antibodies. Lyophilization of the yolks would prolong their shelf life and freeze-dried powder is easier to mix than the fresh yolks.

However, the lyophilisation process resulted in reduced antibody titers in the freeze-dried yolk powder and broilers given this powder were not protected against *Campylobacter* colonization. Earlier, Chalghoumi et al. (2009) and Paul et al. (2014) emphasized that the initial antibody dose administered should be sufficiently high. Indeed, when reducing the administered dose of fresh yolks from 5%, as used during the first study, to 0.5% or 0.05%, no *in vivo* protection was obtained (unpublished results). Secondly, during the passage through the gastrointestinal tract, specific IgY loses activity due to the acidic stomach environment and activities of pancreatic proteases (Schade et al., 2005; Chalghoumi et al., 2009a). The yolk has been hypothesized to form a protective matrix for the antibodies against

degradation and functionality loss during this passage (Schade et al., 2005; Hermans et al., 2014) and this function might be partially lost during the process of freeze-drying. Both the reduction of antibody titers and insufficient protection by the yolk would lead to an insufficient amount of functional antibodies surviving the gastrointestinal passage. *Campylobacter* is transmitted through the flock by the fecal-oral route and contaminated faeces contain high concentrations of *Campylobacter*, resulting in a high environmental infection pressure (Hermans et al., 2012b), which may explain the need for high antibody titers in the gastrointestinal tract. Taken together, our results evidence that freeze-drying of the yolks will not improve applicability of passive immunisation.

Alternatively, the antibodies might be extracted from the yolk before administration through the feed or drinking water (Bizanov, 2017). Since the egg yolk matrix was hypothesized to protect the antibodies against gastrointestinal degradation (Hermans et al., 2014), the antibodies should be processed further to improve their survival.

Although promising results were obtained by prophylactically administering 5% fresh yolk containing bacterin-induced antibodies, this procedure would result in an increase of the production cost from €2.24 to €2.93 per broiler, or an additional estimated annual cost of €8.3 billion in the EU (calculation given in the supplementary notes). The estimated annual cost of human campylobacteriosis in the EU amounts 'only' €2.4 billion (EFSA, 2018) and it is clear that the current method would be too expensive. Moreover, as mentioned above, even additional costs are to be expected, since the yolks should be further processed to prolong shelf life and/or protect the antibodies against gastrointestinal degradation. Neither the producers, nor the consumers, nor the government will be prepared to pay these costs. The question is what maximal additional cost we are prepared to pay. In the end, everyone, especially the consumers themselves but also the government, will benefit when less people catch campylobacteriosis. One could ask oneself why chicken meat, and food in general,

should be as cheap as possible, as if every adaptation to improve for example animal welfare or public health, will a priori be considered to be too expensive. However, some people are prepared to spend more on for example organic products and if a measure can be developed that does not result in a greatly increased production cost, chances are that people might be willing to pay the difference.

Even though eggs will probably not be applied for passive immunisation of broilers against *Campylobacter*, hyperimmune egg yolks can be used for the production of polyclonal antibodies for other purposes, such as research and diagnostics, for example immunologic assays, instead of mammalian IgG. The non-invasive sampling is more animal-friendly and the high yields result in reduced production costs. Companies such as Nabas and Gallus Immunotech provide the possibility to purchase egg yolk-derived IgY and IgY purification kits (Gallus Immunotech Inc., 2019; Nabas, 2019).

In search of an alternative administration method

An alternative to passive immunisation is active immunisation of chickens. An interesting possibility is *in ovo* vaccination, which can be automated and is therefore less labour-intensive and expensive than manual injection (Peebles, 2018). In the third study, broiler embryos were vaccinated with either the bacterin or subunit vaccine. In contrast to the vaccination of the layers in the first study, no antibody response and consequently no *in vivo* protection was obtained. Other researchers did succeed to reduce *Campylobacter* in broilers by experimental *in ovo* vaccination (Kobierecka et al., 2016; Godlewska et al., 2016), although these studies did not yet result in the development of a commercially available vaccine. To improve our *in ovo* delivery method, a carrier can be used, such as the herpesvirus of turkeys (HVT) (Gimeno, 2008), Gram-positive enhancer matrix (GEM) particles and

liposomes (Kobierecka et al., 2016) and *C. jejuni* outer membrane vesicles (OMVs) (Godlewska et al., 2016). Another possibility might be DNA- or RNA-vaccination (Liu et al., 2019).

Other methods to effectively administer the bacterin and subunit vaccine are to be investigated. For example, it was shown that *Campylobacter* infection in layers resulted in delayed infection in their chicks (Sahin et al., 2003). Vaccination of broiler breeder hens against *Campylobacter* using our vaccines might be interesting to investigate. Another possibility to formulate the subunit vaccine antigens is the use of vectors for other administration methods than *in ovo* vaccination. Examples are the ones mentioned above [herpesvirus of turkeys (Gimeno, 2008), GEM particles and liposomes (Kobierecka et al., 2016) and *C. jejuni* OMVs (Godlewska et al., 2016)], *Salmonella* sp. (Wyszyńska et al., 2004; Buckley et al., 2010; Layton et al., 2011; Theoret et al., 2012; Saxena et al., 2013) or *Eimeria tenella* (Clark et al., 2012). A vaccine consisting of a bacterial or viral vector might provide protection against both pathogens (Łaniewski et al., 2014). Alternatively, one could use DNA- or RNA-vaccination (Meunier et al., 2016a; Pardi et al., 2018; Zhang et al., 2019). Finally, other platforms to recombinantly produce the maternal antibodies, such as bacteria, yeast, insect or mammalian cells, and plants, may be used (Schirrmann et al., 2008; Frenzel et al., 2013; Yusibov et al., 2016). Especially plants might be interesting since their seeds can easily be stored and administered through the broilers' feed, and may form a protective matrix for the antibodies against degradation during the gastrointestinal passage (Rademacher et al., 2009; Zimmermann et al., 2009; Yusibov et al., 2016; Vanmarsenille et al., 2018). The disadvantage of recombinant production is that the produced antibodies are monoclonal and a mixture of several recombinant antibodies might be needed to target sufficiently different epitopes, since Campylobacters are highly diverse (Boer et al., 2002; Jerome et al., 2011).

The need for a combined approach

To further reduce campylobacteriosis, other measures should be implemented besides immunisation. Antibiotics cannot be used due to emerging resistance and other alternatives should be further investigated (Dibner and Richards, 2005; Zhu et al., 2006). Feed and drinking water additives, such as fatty and organic acids, are mostly not effective (Hermans et al., 2011a). Phages could be used, however high degrees of resistance rapidly emerge (El-Shibiny et al., 2009; Carvalho et al., 2010; Meunier et al., 2016b) and phages are highly strain specific (Hagens and Loessner, 2010). Bacteriocin application has also been effective (Santini et al., 2010; Svetoch and Stern, 2010), but it is not known if and how fast resistance might develop (Hoang et al., 2011). Competitive exclusion has not been shown to be successful yet (Hermans et al., 2011a).

It has been shown previously that optimization of biosecurity can lead to important reductions in on-farm *Campylobacter* prevalence (van de Giessen et al., 1998; Gibbens et al., 2001; Hald et al., 2007; Rosenquist et al., 2009). These measures include, but are not limited to, control programs for rodents and insects, improved biosafety during or abolishment of partial thinning, improved cleaning and disinfection of the stables and equipment and the proper use of disinfection baths before and after entering a stable. Improving biosecurity can also reduce the prevalence of other pathogens, such as *Salmonella* (Cox and Pavic, 2010), *Eimeria* (Peek and Landman, 2011) and *Mycoplasma gallisepticum* (Evans et al., 2005).

To minimize cross-contamination during slaughter, it is better to focus on optimization of practices, rather than physical or chemical carcass treatment, because of legislation restrictions and consumer acceptance (MacRitchie et al., 2014). This includes improving biosecurity measures, such as proper cleaning and disinfection of the equipment, optimization of processing parameters, such as the scalding temperature, and planning to process

Campylobacter-negative batches before contaminated ones (Seliwiorstow, 2015). The latter, however, can only be applied for one pathogen at a time.

Last but not least, it is important to improve both producer and consumer awareness on this topic. The majority of Belgian farmers visited by Seliwiorstow (2015) were aware of *Salmonella*, but not of *Campylobacter*. Providing correct information to the producers will raise their awareness on this topic and will be necessary to convince them to implement the necessary measures, especially since *Campylobacter* does not seem to influence broiler health and productivity (Seliwiorstow, 2015). Additionally, it is important to inform people about the risks of handling and consuming poultry meat, such as undercooking and cross-contamination in the kitchen (Sampers et al., 2012).

Although researchers have been investigating strategies to reduce *Campylobacter* colonization in chickens for many years, to date measures are limited to preventing *Campylobacter* from entering the flock. Vaccination with whole cell vaccines, attenuated strains or killed bacterins, is complicated by differential expression of surface proteins between the *in vivo* and *in vitro* environment (Panigrahi et al., 1992). It has also been challenging to find suitable antigens for a subunit vaccine. The chicken immune response during infection is mainly directed against flagellin and major outer membrane proteins (MOMPs) (Cawthraw et al., 1994; Widders et al., 1998). However, vaccines against these antigens were not always successful, which can be explained by phase variable glycosylation and alternative expression of flagellin genes (Cawthraw et al., 1994; Khoury and Meinersmann, 1995; Widders et al., 1998). Since results with other antigens were variable, it is important to continue identifying new candidates (Hermans et al., 2014; Meunier et al., 2016c; Mehla and Ramana, 2017). In addition, because broilers are slaughtered at a young age, the birds only have a limited amount of time to develop a protective immune response, further complicating the vaccination strategy.

On the other hand, the issue with oral passive immunisation and other nutritional strategies is that a sufficiently high amount of the active product should survive the gastrointestinal passage and be still functional when reaching its site of action (Wittschier et al., 2007). During colonization, *Campylobacter* hides at the bottom of the mucus layer in crypts and epithelial cells, protected from these compounds (Van Deun et al., 2008b; Hermans et al., 2010; Hermans, 2012). This could explain why many of these compounds seem promising *in vitro* but disappoint when tested *in vivo*. Premature degradation can be overcome by protecting the compound by coating on or encapsulating in a carrier (Van Immerseel et al., 2004). Many aspects of the exact colonization mechanism by *Campylobacter* and its interaction with the chicken host and host microbiome remain unknown. More research will certainly be needed to fully understand these mechanisms. This knowledge could ultimately contribute to the development of an effective control strategy.

Taken together, many uncertainties remain which methods would be most effective and affordable. Factors that certainly need to be addressed are biosafety, optimization of the slaughter process and informing both producers and consumers. However, more research will be needed before a combined action plan, including for example immunisation, can be developed.

Conclusion and future perspectives

This thesis was focused on developing an immunisation strategy to control *Campylobacter* in broiler chickens. We have composed two vaccines with a broad reactivity to heterogeneous *Campylobacter* strains that induced protective antibodies, when applied for passive immunisation. Future research could benefit from these results by applying the vaccines for other immunisation strategies, such as optimized *in ovo* vaccination or breeder vaccination.

On the other hand, the subunit vaccine antigens could be used for DNA or RNA vaccination, or the antibodies could be produced recombinantly, for example in plant seeds, for oral administration.

Next to the development of an effective immunisation strategy, biosecurity measures should be optimized and possibly other methods could be applied as well. When combined, these measures may contribute to *Campylobacter* control in poultry and lead to a reduction in the number of human campylobacteriosis cases.

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SUPPLEMENTARY NOTES

Calculation of the estimated additional annual cost of the developed method

To estimate the costs for passive immunisation of broiler chicks against *Campylobacter* sp., the additional cost for treatment of the chicks with fresh egg yolks from bacterin or subunit vaccinated layer hens during the full production period (38 days) was calculated.

The price for already registered bacterins and subunit vaccines varies between 4-20 eurocent per vaccination dose (Medini, Oostkamp). A similar price range can be expected for a commercial *Campylobacter* vaccine. For the vaccination scheme used in this research (4 vaccinations), this would result in 16-80 eurocent per layer hen. A layer hen produces approximately 420 eggs (Lohmann, 2017), resulting in an additional cost of 0.04-0.19 eurocent per egg. The mean production cost per egg in 2018 was 5.32 eurocent (pluimvee.be, accessed at 13/5/2019). In total, this would result in a maximal cost of 5.51 eurocent per egg.

A broiler chicken consumes 4 kg of feed during the production period (Aviagen, 2017). When administering 5% hyperimmune egg yolk, 200 g of yolk will be needed per chicken. Since one yolk weighs 16 g, 12.5 yolks would be needed per broiler chick, resulting in a cost of 0.69 euro per chicken.

In 2018, a farmer received 0.89 euro per kg broiler (pluimvee.be, accessed at 7/5/2019). A chicken of 2.52 (Aviagen, 2017) would result in 2.24 euro. Because of the additional cost of the hyperimmune yolk, this price would increase by 30.71% to 2.93 euro.

In 2017, 12 billion broilers were produced in the EU (USDA Foreign Agricultural Service, 2017), resulting in a cost of 8.3 billion euro to treat the broilers with our method. For comparison, the annual cost of campylobacteriosis to public health and loss of productivity in

the EU is estimated at 2.4 billion euro (https://www.efsa.europa.eu/en/topics/topic/campylobacter, accessed at 7/5/2019). The treatment would cost three or four times as much as the most optimal benefits.

The largest share of the costs can be attributed to the use of eggs. Reducing the number of eggs needed for example by delaying the start of the treatment for two weeks, would make little difference since the broilers consume the most feed at the end of the production period. Moreover, the yolks will need to be processed further since the administration of fresh yolks is not applicable, which would further increase the costs.

Therefore, the use of hyperimmune egg yolks would be too expensive.

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SUMMARY

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Consumption of poultry meat contaminated with *Campylobacter* remains one of the main sources of foodborne gastrointestinal illness. Carcass and meat contamination originates from cecal *Campylobacter* colonization in the birds. To date, no effective control measures exist to reduce cecal *Campylobacter* colonization in chickens. Previously, passive immunisation of broiler chicks by oral administration of egg yolk antibodies against a whole cell *Campylobacter jejuni* vaccine was shown to protect the birds against *C. jejuni* colonization. The aim of this project was to study the efficacy and applicability of passive and active immunisation against *C. jejuni* and *C. coli* in broilers, using two novel vaccines, a bacterin mix of thirteen *C. jejuni* and *C. coli* strains and a subunit vaccine consisting of six immunodominant *C. jejuni* proteins.

In the first study, the bacterin and subunit vaccines were applied for passive immunisation. Vaccination of layer hens resulted in a high and prolonged immune response, detected as *Campylobacter*-specific IgY in their egg yolks. These antibodies, especially when bacterin-induced, were shown to react to heterogeneous *C. jejuni* and *C. coli* strains. Prophylactic administration of the fresh hyperimmune egg yolks through the broilers' feed, significantly reduced the percentage of *C. jejuni*-colonized birds after challenge from 78% to 15% (bacterin) and 44% (subunit vaccine). When administered therapeutically, the bacterin yolk antibodies were able to significantly reduce cecal *Campylobacter* numbers in infected birds by approximately 1 $\log_{10}$. In both experiments, better results were obtained using the bacterin-induced antibodies. Combined with the broader reaction to heterogeneous *Campylobacter* strains, follow-up experiments preferably apply prophylactic administration of the bacterin treatment.

The addition of fresh yolks to broiler feed will not be applicable in the field because of the short conservation and high viscosity, complicating mixing the yolks through the feed.

Therefore, it was investigated if freeze-dried yolks would offer a similar level of protection against *Campylobacter* colonization in broilers. Unfortunately, this was not the case. The lyophilisation process resulted in a 16-fold reduction of antibody titers in the freeze-dried yolk powder, which might (partially) explain the lack of *in vivo* protection. Other authors emphasized before that the initial antibody dose administered should be sufficiently high, which was also found in our own unpublished experiments. Secondly, fresh yolk was hypothesized to form a protective matrix for the antibodies during the gastrointestinal passage and this function might be partially lost during the process of freeze-drying. Both the reduction of antibody titers and insufficient protection by the yolk would lead to an insufficient amount of functional antibodies surviving the gastrointestinal passage and freeze-drying of the yolks will not make passive immunisation applicable.

Promising results were obtained in the first study for passive immunisation, however, this procedure would result in an estimated increase of the production cost of €8.3 billion per year in the EU, which would be too expensive. Therefore, active immunisation through *in ovo* vaccination using the bacterin and subunit vaccine was investigated. No antibody response was induced in the vaccinated embryos, in contrast to the layers in the first study, and consequently no *in vivo* protection was obtained in the broilers after challenge with *C. jejuni*. Since other authors did succeed in reducing *Campylobacter* colonization after *in ovo* vaccination, optimization of the vaccines might be necessary. Alternatively, there are still other administration methods to be explored, such as broiler breeder vaccination, the use of vectors or DNA/RNA-vaccination, or other platforms to recombinantly produce the maternal antibodies. To minimize *Campylobacter* occurrence in poultry, immunisation should certainly be combined with other measures, such as (but not limited to) biosafety and optimization of slaughterhouse practices.

In conclusion, we have composed two vaccines with a broad reactivity to heterogeneous *Campylobacter* strains, inducing protective antibodies when applied for passive immunisation. Future research could benefit from these results by applying the vaccines for other immunisation strategies, which ultimately may contribute to *Campylobacter* control in poultry and a reduction in the number of human campylobacteriosis cases.

SAMENVATTING

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Het consumeren van gevogelte besmet met *Campylobacter* blijft één van de belangrijkste oorzaken van gastro-intestinale voedselinfectie. Deze contaminatie wordt veroorzaakt door de kolonisatie van de ceca door *Campylobacter*, maar tot op heden bestaan er geen efficiënte controlemaatregelen om *Campylobacter* in kippen te bestrijden. In een eerdere studie werd aangetoond dat braadkippen beschermd waren tegen kolonisatie door *C. jejuni* door het toepassen van passieve immunisatie, meer bepaald door de orale toediening van eierdooier die antistoffen bevatte tegen een *whole cell Campylobacter jejuni*-vaccin. Het doel van dit project was om de werkzaamheid en toepasbaarheid van passieve en actieve immunisatie tegen *C. jejuni* en *C. coli* na te gaan, ditmaal met twee nieuwe vaccins: een bacterin samengesteld uit dertien *C. jejuni*- en *C. coli*-stammen en een subunitvaccin samengesteld uit zes immunodominante *C. jejuni* eiwitten.

In de eerste studie werden het bacterin en subunitvaccin toegepast voor passieve immunisatie. Vaccinatie van leghennen resulteerde in een hoge en langdurige immuunrespons, gemeten als *Campylobacter*-specifieke IgY in de eierdooiers van de hennen. Van deze antistoffen, in het bijzonder de bacterin-geïnduceerde antistoffen, werd aangetoond dat ze reageerden tegen heterogene *C. jejuni*- en *C. coli*-stammen. Na profylactische toediening van de verse hyperimmune dooiers doorheen het voeder van kuikens bleek het percentage *C. jejuni*-gekoloniseerde dieren na inoculatie significant verlaagd te zijn van 78% naar 15% (bacterin) en 44% (subunit vaccin). Wanneer de antistoffen therapeutisch toegediend werden, bleken de bacterin-geïnduceerde antistoffen de cecale *Campylobacter*-aantallen in geïnfecteerde dieren significant te verlagen met ongeveer 1 $\log_{10}$. In beide experimenten werden betere resultaten behaald met de bacterin-geïnduceerde antistoffen. Aangezien deze ook een bredere reactie tegen de heterogene *Campylobacter*-stammen vertoonden, is het aan te raden om in

vervolgexperimenten de bacterin-geïnduceerde antistoffen te gebruiken en deze profylactisch toe te dienen.

De toediening van verse dooier-antistoffen aan het voeder van braadkuikens is niet toepasbaar in de praktijk door de korte bewaartijd en hoge viscositeit van de dooiers, die het mengen doorheen het voeder bemoeilijkt. Daarom werd onderzocht of gevriesdroogde dooiers een gelijkaardige bescherming kunnen bieden tegen *Campylobacter*-kolonisatie in kippen. Helaas bleek dit niet het geval te zijn. Het lyofilisatieproces resulteerde in een 16-voudige daling van de antistoftiters in het gevriesdroogde dooierpoeder, wat (gedeeltelijk) het gebrek aan *in vivo*-bescherming kan verklaren. Andere auteurs hebben reeds benadrukt dat de initieel toegediende dosis voldoende hoog moet zijn, wat ook bevestigd werd door onze eigen ongepubliceerde resultaten. Ten tweede wordt verondersteld dat de verse dooier een beschermende matrix vormt voor de antistoffen tijdens de gastro-intestinale passage en deze functie zou gedeeltelijk verloren gegaan kunnen zijn gedurende het vriesdroogproces. Zowel de verlaagde antistoftiter als het verlies van deze bescherming zouden leiden tot een onvoldoende groot aantal functionele antistoffen die de gastro-intestinale passage overleven. Vriesdrogen van de dooiers zal de passieve immunisatie dus niet toepasbaar maken.

Tijdens de eerste passieve-immunisatiestudie werden veelbelovende resultaten behaald. Deze procedure zou echter een verhoogde productiekost met zich meebrengen, geschat op €8.3 miljard per jaar in de EU, wat te duur zou zijn in vergelijking met de huidige kosten ten gevolge van humane campylobacteriose. Daarom werd tijdens de laatste studie actieve immunisatie, door *in ovo*-vaccinatie met het bacterin en subunitvaccin, onderzocht. In tegenstelling tot de vaccinatie van de leghennen in de eerste studie, werd geen antistofrespons geïnduceerd in de gevaccineerde embryo's en werd bijgevolg geen *in vivo*-bescherming bekomen in de kuikens na inoculatie met *C. jejuni*. Aangezien andere auteurs er wel in slaagden om kolonisatie door *Campylobacter* te verminderen na *in ovo*-vaccinatie, is het

noodzakelijk om de vaccins te optimaliseren. Een alternatief is om andere toedieningsmethoden te onderzoeken, zoals vaccinatie van de ouderdieren van de braadkuikens of het gebruik van vectoren, DNA- of RNA-vaccinatie, of andere platformen voor recombinante productie van de maternale antistoffen. Om de *Campylobacter*-prevalentie in pluimvee te minimaliseren, zal immunisatie zeker gecombineerd moeten worden met andere maatregelen, zoals (maar niet beperkt tot) bioveiligheid en optimalisatie van de slachthuispraktijken.

Samengevat werden twee vaccins ontworpen met een brede reactiviteit tegenover heterogene *Campylobacter*-stammen, die beschermende antistoffen opwekken wanneer ze toegepast worden voor passieve immunisatie. In de toekomst kan op deze resultaten verder gebouwd worden door de vaccins toe te passen voor andere immunisatiestrategieën, die uiteindelijk kunnen bijdragen aan de controle van *Campylobacter* in pluimvee en een verlaging van het aantal humane campylobacteriosegevallen.

CURRICULUM VITAE

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Jasmien Vandeputte werd geboren op 1 oktober 1993 in Gent. Na haar middelbare studies Wetenschappen-Wiskunde, startte ze in 2011 met de opleiding Bio-Ingenieurswetenschappen, afstudeerrichting Cel- en Genbiotechnologie aan de Universiteit Gent, die ze in 2016 succesvol afrondde met onderscheiding.

Vanaf oktober 2016 trad ze in dienst als doctoraatsbursaal aan de vakgroep Pathologie, Bacteriologie en Pluimveeziekten, waar ze gedurende drie jaar onderzoek verrichtte naar de immunisatie van pluimvee tegen *Campylobacter*. Ze is auteur van meerdere publicaties in internationale tijdschriften en presenteerde haar onderzoeksresultaten op een internationaal congres.

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PUBLICATIONS

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CONFERENCE CONTRIBUTIONS

Garmyn, A., **J. Vandeputte**, A. Martel, S. Canessa, N. Van Rysselberghe, L. De Zutter, M. Heyndrickx, F. Haesebrouck, F. Pasmans, and A. Garmyn. 2019. Reduction of *Campylobacter jejuni* colonization in broilers by in-feed supplementation with hyperimmune egg yolk antibodies. Poster for the 6th International conference on poultry intestinal health, April 2-5, Rome, Italy.

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DANKWOORD

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