Campylobacter colonization in broilers

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Chapter 1

General introduction

Foodborne diseases are a major public health concern worldwide, and encompass a wide spectrum of - mainly gastroenteric - illnesses, caused by viral, bacterial, parasitic or chemical contamination of food (EFSA, 2009). The contribution of contaminants to the occurrence of disease vary substantially between countries, but *Campylobacter* spp. are considered to be a major cause of diarrheal illness in humans and are generally regarded as the most common bacterial cause of foodborne disease worldwide.

CAMPYLOBACTERIOSIS IN HUMANS

Symptoms

Currently, the genus *Campylobacter* consists of 17 species (Debruyne et al., 2008), of which the most frequently reported in human cases are *Campylobacter jejuni* (*C. jejuni* subspecies *jejuni*) and *Campylobacter coli* (Blaser et al., 2008; Lastovica et al., 2008). The most prominent clinical manifestation of campylobacteriosis in humans is enteritis. The onset of disease is often acute with abdominal cramps, shortly followed by diarrhea. Other symptoms in order of decreasing frequency are fever, headache, myalgia, blood in feces, and vomiting (Blaser et al., 2008). The majority of patients recover within a week after onset of the symptoms, but some develop complications after the acute phase. A few, usually elderly patients, may die due to the infection. Approximately 1 in 1000 patients develops Guillain-Barré Syndrome, an autoimmune-mediated neurological disorder (Nachamkin et al., 2000). Other complications of campylobacteriosis may be Reactive Arthritis and Post Infectious Irritable Bowel Syndrome (PI-IBS) (DuPont, 2008; Schaad, 1982).

Incidence rate and disease burden

The diagnosis of campylobacteriosis is confirmed by isolating *Campylobacter* from stool samples of patients. Many patients, however, do not visit their general practitioner or are not requested to provide a stool sample. Because of this incomplete reporting, the incidence rate of campylobacteriosis is difficult to determine. In studies in England (Wheeler et al., 1999) and the Netherlands (de Wit et al., 2001) the phenomenon of underreporting was investigated, resulting in estimates of 7.6 and 19 cases falling ill per reported case, respectively. The accompanying estimates of the incidence rate of *Campylobacter*-associated illnesses were 870 and 916 per 100,000 inhabitants *per annum* in England and the Netherlands, respectively.

To determine the disease burden of campylobacteriosis, information on both the incidence rate and the severity of disease is required. Disease burden is often expressed in numbers of disability adjusted life years (DALYs), allowing comparison for different populations and diseases. DALYs express the years of life lost to premature death (YLL) and years lived with disability (YLD) weighted with a factor between 0 and 1 for the severity of the disability. Considering foodborne pathogens in the Netherlands in 2004, toxoplasmosis caused the largest disease burden (2,400 DALYs per year, on a population of 16 million), and campylobacteriosis was the second most contributing disease, with a total estimate of 1,300 DALYs, followed by salmonellosis (500 DALYs) (Kemmeren et al., 2006). The main contribution to the total estimate for campylobacteriosis is from late-onset manifestations of *Campylobacter* infections, in particular Guillain-Barré syndrome and PI-IBS (640 DALYs). Mortality is the second largest contributor (390 DALYs), while uncomplicated gastroenteritis accounts for 179 DALYs (Kemmeren et al., 2006). The total costs of illness are estimated at € 19.6 million *per annum* in the Netherlands (Kemmeren et al., 2006).

Risk factors for human infection

In several field studies, human exposure to sources and potential risk factors have been investigated. These studies consistently indicated several routes of infection: consumption of and contact with poultry, contact with pets and non-poultry farm animals, and drinking surface water or raw milk. Additionally, traveling to foreign countries was associated with campylobacteriosis (Olson et al., 2008). In Danish and Australian case-control studies, the proportion of human cases attributed to the consumption of poultry meat was estimated at 23.8% and 29.3%, respectively (Stafford et al., 2008; Wingstrand et al., 2006). These proportions should, however, be interpreted carefully, as bias by selective recall, and prior population immunity can occur (Adak et al., 1995).

In a recent study, human isolates were genotyped, using Multi Locus Sequence Typing (MLST), and compared to sequence-types of isolates from wild birds, domestic animals (chicken, cattle, sheep, pig, rabbit) and the environment (sand and water) to assess the quantitative contribution of these sources (Wilson et al., 2008). In this study, chicken contributed to 56.5% of human cases, followed by cattle (35.0%) and sheep (4.3%). The contribution of foodborne transmission can, however, not be determined in such studies.

Although its contribution cannot be quantified precisely, poultry is generally considered the most important source of foodborne campylobacteriosis.

Mishandling resulting in cross-contamination of other foods is considered the most important route of infection (Luber, 2009). Interventions aimed at reducing poultry-related exposure of *Campylobacter* to humans are, therefore, likely to reduce the incidence rate, and the disease burden, of human campylobacteriosis.

CAMPYLOBACTER IN POULTRY

All types of poultry (e.g. broilers, layers, turkeys, and ducks) can become colonized with *Campylobacter* (Yogasundram et al., 1989). Up to now, *Campylobacter* spp. have not been recovered from eggs originating from *Campylobacter*-positive flocks (Sahin et al., 2003). Consequently, poultry meat is considered the product to be responsible for foodborne transmission of *Campylobacter*. Within the poultry industry, broilers account for the largest poultry meat market worldwide.

Colonization of broilers

Campylobacter spp. do not evoke clinical signs or pathological lesions in broilers (Dhillon et al., 2006). The bacteria predominantly colonize the ceca, and reside in the intestinal mucous layer over the intestinal crypts of the villi (Beery et al., 1988), and translocation to internal organs is commonly observed (Cox et al., 2005; Cox et al., 2006). Chickens are susceptible to colonization, with inoculation doses as low as 35-40 colony forming units (cfu) resulting in colonization (Cawthraw et al., 1996; Stern et al., 1988). Colonization, occurring as early as one day after inoculation of high doses of *Campylobacter* (Knudsen et al., 2006; Shanker et al., 1988), results in maximal levels in the ceca after 5 days (8-9 ^{10}Log cfu/g). A slight decline of colonization levels occurs after about 4 weeks, and birds with undetectable levels may then occur (Achen et al., 1998). The majority of broilers, however, will shed *Campylobacter* in concentrations above 6 ^{10}Log cfu/g feces for the remaining rearing period (El-Shibiny et al., 2005; Knudsen et al., 2006). *C. jejuni* is the most commonly isolated *Campylobacter* species in colonized broilers, (Evans et al., 2000) and on broiler meat products (Nielsen et al., 2006). *C. coli* was isolated in 11% of colonized flocks in this field study (Evans et al., 2000). In free range and organic broiler flocks, however, *C. coli* is increasingly dominant from 5 weeks of age (El-Shibiny et al., 2005).

Colonization of broiler flocks

On commercial broiler farms, *Campylobacter* is rarely detected in birds younger than 2 - 3 weeks (Bull et al., 2006; Gregory et al., 1997; Jacobs-Reitsma et al., 1995; Ring et al., 2005). From 2 weeks until slaughtering age (5-8 weeks), the probability of becoming colonized increases (Bouwknegt et al., 2004; Evans et al., 2000). The prevalence of *Campylobacter*–positive flocks at slaughtering age varies with outdoor temperature (Hartnack et al., 2008; Patrick et al., 2004), with lower proportions in winter compared to summer. There appears to be a consistently reported lower prevalence of positive flocks in the north of Europe compared with central European countries like the United Kingdom and The Netherlands (Saleha et al., 1998). The current explanations for this phenomenon are ambient temperature, humidity, farm density, and the presence of vectors such as flies. Other factors, e.g. age at slaughtering, might also be responsible for this difference.

Risk factors for colonization of broiler flocks

During the last two decades, field studies have provided knowledge on risk factors for colonization of commercial broiler flocks. The most relevant risk factors are the presence of multiple poultry houses (Bouwknegt et al., 2004; McDowell et al., 2008), the absence of hygiene barriers (Cardinale et al., 2004; McDowell et al., 2008), and the presence of other farm animal or pets (Bouwknegt et al., 2004; Cardinale et al., 2004). Partial depopulation (or thinning) of flocks has also been considered an important risk factor (Adkin et al., 2006), but conflicting study results do exist (Hald et al., 2000; Russa et al., 2005), which might have been caused by variation in the duration of the remaining rearing period after thinning or the lack of data on the colonization status of involved flocks at the moment of thinning. Therefore, for now it is unclear to which extent thinning contributes to colonization of broiler flocks.

Although risk factors have been identified, the exact routes of transmission remain largely unknown. Therefore, molecular epidemiology is increasingly used to reveal genetic relationships of isolates detected in flocks and in the farm environment (Ogden et al., 2007; Ridley et al., 2008; Zweifel et al., 2008). It is however difficult to verify whether environmental contamination should be considered a source for or a result of flock colonization, because recently colonized flocks can remain undetected due to the initial low prevalence of shedding birds (Johnsen et al., 2006; Ridley et al., 2008). If the moment at which *Campylobacter* is introduced in the flock was known, it would be easier to verify whether environmental contamination is a cause or a consequence. Tools for the estimation of the moment

of introduction should therefore be developed to assess the contribution of potential sources to flock colonization.

Intervention measures to reduce poultry-related human exposure
Considering the diversity of risk factors for broiler colonization and the high incidence of positive flocks, improving kitchen hygiene by education of consumers would be a rational strategy in the prevention of poultry-related campylobacteriosis. Under laboratory conditions, kitchen interventions (i.e. replacing cutlery and cutting board after handling raw chicken and prevention of hand contact) have indeed shown to result in significantly reduced cross-contamination (Verhoeff-Bakkenes et al., 2008). Consumer-style cleaning of cutlery, cutting board, and hands is, however, ineffective (de Jong et al., 2008) and consumer education is unlikely to result in decreased cross-contamination (Cogan et al., 1999). Therefore, an intervention policy primarily aimed at education of consumers is unlikely to result in reduced incidence rates of human campylobacteriosis.

As contamination of broiler meat is a result of cross-contamination from the gut and the skin of colonized broilers (Rasschaert et al., 2006), reducing the concentration of *Campylobacter* in the gut of colonized broilers, improving slaughtering techniques and decontamination of carcasses, might be another strategy in the prevention of campylobacteriosis.

Pre-slaughter treatments of positive broilers have resulted in a reduction of the intestinal concentration of *Campylobacter* (de los Santos et al., 2009; Loc Carrillo et al., 2005; Wagenaar et al., 2005). It is, however, unclear whether this decrease can also be realized under commercial conditions and whether decreased colonization levels result in decreased carcass contamination levels (Stern et al., 2003).

Prevention of rupture of intestines during evisceration and removing the gut content of the lower intestines prior to defeathering have been suggested to reduce carcass contamination levels (Rosenquist et al., 2007). However, equipment for these operations is difficult to develop due to the different sizes of carcasses and variation in the position of the gut. Whether future techniques will result in decreased cross-contamination is unclear for now.

Various chemical and physical methods for carcass decontamination of broiler carcasses have been evaluated in the last decade (Bauermeister et al., 2008).

Chemicals may reduce the concentration of *Campylobacter* by 1-2 log units (or 90-99%) (Rosenquist et al., 2007). At present, chemicals, such as sodium hypochlorite and peroxyacetic acid, which are routinely used in the USA, have not been approved for carcass decontamination in the EU. Of all techniques for physical contamination that have been studied (e.g. freezing, crust-freezing, hot water washing), freezing is considered the most effective one, with a 1-2 log units reduction of contamination levels depending on the duration of frozen storage (Georgsson et al., 2006; Rosenquist et al., 2007). Physically decontaminated meat can however not be sold as fresh products. This implies that flocks would have to be withheld from the fresh poultry meat market, especially in seasons or regions with a high incidence of positive broiler flocks. As most countries have incidence rates that exceed the demand for processed poultry meat, the incidence should decrease significantly to allow decontamination to be applied on all positive flocks.

All research carried out so far, and all control measures implemented up to now have resulted in a slight reduction of human exposure, but human campylobacteriosis is still occurring, and the number of *Campylobacter*-positive broiler flocks seems to remain stable.

SCOPE AND OUTLINE OF THIS THESIS

Scope

Measures to reduce human exposure can be applied in kitchens, slaughterhouses and poultry farms. The development and optimization of control strategies requires knowledge of the epidemiology of *Campylobacter* both in humans and in poultry. The research in this thesis focused on the epidemiology of *Campylobacter* in the primary sector, i.e. the broiler flock.

Outline

Considering all limitations and uncertainties regarding previously discussed interventions, reducing the incidence of *Campylobacter*-positive flocks would have the most pronounced effect on the incidence of human campylobacteriosis. This can be realized by prevention of exposure or decreasing the birds' susceptibility for *Campylobacter* colonization. Since the most important risk factors for colonization of commercial broilers (Adkin et al., 2006) appear to relate to the introduction of contaminated persons, materials or insects into the broiler house, prevention of introduction should be the first aim. The exact routes of introduction remain, however, unclear. To identify these routes, information on the moment of

introduction would be useful. It seems, however, unfeasible to detect the first bird that is colonized in a commercial broiler flock because of flock size and the necessary sampling frequency. To determine the moment of introduction, knowledge on the dynamics of *Campylobacter* colonization within a flock is required. In **chapter 2** transmission of *Campylobacter* was modeled in experimentally exposed groups of broilers. As experimental conditions differ from the field situation, the transmission rate in commercial flocks might differ from the experimentally derived transmission rates, and might vary between flocks. Therefore, in **chapter 3**, the distribution of transmission rates of *Campylobacter* under field conditions was estimated using the same mathematical model. Using this distribution the moments of introduction was estimated in the study-population.

Even if most introduction routes were known, introduction of *Campylobacter* can probably not be prevented completely under field conditions. Therefore, interventions that can decrease the broiler's susceptibility for *Campylobacter* colonization would be beneficial in the prevention of broiler colonization. To develop and evaluate potential interventions, knowledge on the relation between the *Campylobacter* exposure dose and the probability of colonization is required. In **chapter 4**, an experimental *in vivo* colonization model was used to assess this relation in broilers. Additionally, the effect of an intervention was assessed.

If, despite all effort, flock colonization cannot be prevented, reduction of the colonization levels in broilers at slaughtering might reduce carcass contamination, and subsequently, human exposure. In **chapter 5** a broiler colonization model was used to study the effect of pre-slaughter treatment on colonization levels in the digestive tract of broilers. Additionally, correlation between colonization levels in different parts of the digestive tract was assessed.

In vivo colonization studies are costly and elaborate, and require the use of many broilers. The development of alternative models that mimic *Campylobacter* colonization in broilers could limit the use of experimental animals and costs. Broiler embryos at the last stage of incubation have developed a functional gastrointestinal tract (Jochemsen et al., 2002; Yoshizaki et al., 2002) and might be suitable as an alternative colonization model. Therefore, in **chapter 6** the *Campylobacter* colonization characteristics and the dose response relation of strains with varying *in vivo* colonization capacities were explored in broiler embryos.

In the process of *Campylobacter* colonization in broiler flocks various steps can be distinguished, at which control strategies can be targeted. In the general discussion (**chapter 7**) the methods and newly acquired information described in this thesis are used to discuss current knowledge on interventions and methods to evaluate interventions for each of these steps.

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Chapter 2

Quantifying transmission of *Campylobacter* spp. among broilers

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ABSTRACT

Campylobacter species are frequently identified as a cause of human gastroenteritis, often from eating or mishandling contaminated poultry products. Quantitative knowledge of transmission of *Campylobacter* in broiler flocks is necessary, as this may help to determine the moment of introduction of *Campylobacter* in broiler flocks more precisely. The aim of this study was to determine the transmission rate parameter in broiler flocks. Four experiments were performed, each with four *Campylobacter*-inoculated chicks housed with 396 contact chicks per group. Colonization was monitored by regularly testing fecal samples for *Campylobacter*. A mathematical model was used to quantify the transmission rate, which was determined to be 1.04 new cases per colonized chick per day. This would imply that, for example, in a flock of 20,000 broilers, the prevalence of *Campylobacter* would increase from 5% to 95% within 6 days after *Campylobacter* introduction. The model and the estimated transmission rate parameter can be used to develop a suitable sampling scheme to determine transmission in commercial broiler flocks, to estimate whether control measures can reduce the transmission rate, or to estimate when *Campylobacter* was introduced into a colonized broiler flock on the basis of the time course of transmission in the flock.

INTRODUCTION

Campylobacter species are often identified as bacterial causes of human gastroenteritis throughout the world (Ahmed et al., 2002). As an important source of human infections is the mishandling and consumption of contaminated poultry meat (Saleha et al., 1998), a reduction in poultry meat contamination might reduce human exposure and, consequently, the risk of campylobacteriosis.

Several control measures have been implemented to reduce the exposure of humans to *Campylobacter* spp., either by reducing the incidence of *Campylobacter* infections in broiler flocks by biosecurity measures at farms or by improving slaughterhouse hygiene. However, these measures are apparently not sufficiently effective, because many broiler flocks still become colonized with *Campylobacter* spp. (Anonymous, 2003). Therefore, intervention strategies should be improved or alternatives developed.

Current intervention strategies are based on risk factors identified in field surveys (Bouwknegt et al., 2004; Evans et al., 2000; van de Giessen et al., 1998). An important disadvantage of these surveys is that they used associative static models to determine an association between risk factors and the presence of *Campylobacter* in a flock and were based on qualitative data on the infection status of the flocks at the end of the production period. These studies did not take the dynamic aspects of a *Campylobacter* infection in a flock into account. Quantitative knowledge of the transmission of *Campylobacter* is important for the development of control programs for various reasons (Cawthraw et al., 1996). First, it enables us to determine which measures can reduce transmission, and to what extent (de Jong, 1995). Secondly, the transmission rate affects the prevalence of an infection in a population in time, which, in turn, determines the probability of detection. Finally, it may help to determine the moment of introduction of *Campylobacter* in commercial broiler flocks under field conditions (Harrington et al., 2003; Heres et al., 2004; Shanker et al., 1990). With this knowledge, control measures could focus more on high-risk periods, which might facilitate the maintenance of biosecurity measures at the appropriate level.

Clear quantitative information on *Campylobacter* transmission is still lacking, although some transmission experiments have been carried out (Shanker et al., 1990; Stern et al., 1988). Unfortunately, transmission in these studies was only determined qualitatively. Hartnett et al. (2001) analyzed the experiments and did quantify transmission, but their exact method of analysis is unclear. Data from the

study of Jacobs-Reitsma (1996) were available for further analysis. That group carried out four experiments to determine whether groups of 400 broilers could be colonized after introduction of a few *Campylobacter*-inoculated seeder birds. This experimental setup, with four seeder birds per group, a high sampling frequency scheme, and relatively large sample sizes, offered the opportunity to quantify transmission. Here, we present the results of a further quantitative analysis of these data (Jacobs-Reitsma, 1996) and a quantification of the transmission using a mathematical model. These models can be useful in unraveling complex processes at the population level by clarifying some of the factors that determine the speed and scale of transmission of an infectious disease (Anderson et al., 1992; Diekmann et al., 2000; May et al., 1987). In addition, we show how the transmission parameter could be used to estimate the moment of *Campylobacter* introduction in the field and how the precision of this estimation is affected by the sampling scheme and sample size.

MATERIALS AND METHODS

Animals

In four experiments (numbers 1 to 4), the horizontal spread of *Campylobacter* among broilers (type Ross) was studied. Day-old chicks, used in experiments 1 and 2, were obtained from a *Campylobacter*-free parent flock of 2,870 birds housed at the Centre for Poultry Research and Information Services "het Spelderholt" (Beekbergen, The Netherlands). This flock was tested for the presence of *Campylobacter* spp. in 14 pooled samples (four cecal droppings per pooled sample) at day 7 after egg collection. No *Campylobacter*-positive samples were found after 48 h of culture (method described in Jacobs-Reitsma et al., 1995b). The chicks used in experiments 3 and 4 originated from a commercial parent flock which was colonized with *Campylobacter* spp.

Housing

In each experiment, 400 broilers were accommodated at day of hatch in a separate shed at a density of 20 broilers per m^2, which is similar to the housing density under commercial conditions. The broilers were fed commercial broiler feed. They were housed on wood shavings, and the drinking water was supplied by means of a nipple drinking system. Before the start of the experiments, samples were taken from water, feed, and wood shavings in the broiler sheds and tested for *Campylobacter*. The box liners used in the hatchery were tested for *Campylobacter* as well.

Inoculation

The *Campylobacter* strains and inoculation doses are listed in Table 1. *Campylobacter coli* strain C136 was isolated from a pig farm in March 1990 (Heres et al., 2004). *Campylobacter jejuni* strain C356 was isolated in 1990 from boilers (Penner serotype O2) (Jacobs-Reitsma et al., 1995a) and registered in the CAMPY-NET reference set as number CN076 (Harrington et al., 2003). The strains are stored in glycerol at - 80°C and have often been used by the Animal Sciences Group in Lelystad for infection experiments and as reference control strains (de Boer et al., 2000). C. *jejuni* strain C4021 (experiment 4) originated from the parent flock of the chicks.

The strains were freshly cultured in heart infusion broth (microaerobically, 37°C, overnight) and diluted in saline to obtain the intended inoculation dose. The actual concentration (cfu/ml) of *Campylobacter* in the administered suspensions was determined by plating on cephoperazone charcoal desoxycholate agar (CCDA). The complete medium consisted of *Campylobacter* blood-free selective agar base medium (Oxoid CM739) plus CCDA selective supplement SR155.

Table 1. Challenge strains and inoculation dose

Expt	*Campylobacter* strain	Penner serotype	Dose (cfu/broiler)
1	*C. coli* 136	O:46	6.5×10^8
2	*C. coli* 136	O:46	6.5×10^8
3	*C. jejuni* 356	O:2	2.6×10^4
4	*C. jejuni* 4021	Not determined	1.1×10^5

Experimental design

In experiments 1 and 2, four chicks per group were orally inoculated with 0.1 ml of the *Campylobacter* inoculation suspension at day of hatching. In experiments 3 and 4, four chicks per group were orally inoculated with 0.1 ml of the *Campylobacter* inoculation suspension 1 day after hatching. The inoculated chicks (seeders) were marked on the head with a black spot, were given an identification wing number, and were placed back into their shed. All experiments lasted 42 days. The use of four seeders increases the probability that a large outbreak will occur, allowing

transmission to be quantified more accurately (Diekmann et al., 2000). The experiment was approved by the Animal Care and Use Committee (license number 44600).

Sampling

The chicks were sampled at fixed time points (Table 2), starting 1 day after inoculation. In experiments 1 and 2, the four seeders and 50 chicks, chosen at random, were removed from the groups for sampling for *Campylobacter* by cloacal swabbing. After sampling, the broilers were put back into their groups. In experiments 3 and 4, the seeders were removed from the groups for the time necessary to obtain a fresh (cecal) dropping. A swab was taken from these droppings. Fifty samples of soft, fresh, wet, and homogeneous cecal droppings were collected from the broiler sheds, which were divided into five sectors (1 by 4 m each). Defecation was stimulated by turning on the lights and making a noise, which ensured the samples were fresh. When all samples appeared to be *Campylobacter*-positive, the sample size was reduced in all four experiments to 10 or 12 per group.

Samples were collected with sterile swabs and transported to the laboratory in modified Amies transport medium without charcoal (Probact transport swabs; Technical Service Consultants Ltd., United Kingdom). Swabs were directly streaked on CCDA (Oxoid CM739 plus SR155), incubated microaerobically at 42°C for 2 days, and examined for the presence of *Campylobacter*. The rest of the material on the swabs was pooled for the seeders and contact birds separately and enriched. In experiments 3 and 4, the samples of the seeders were enriched individually. Enrichment was in CCD broth (Oxoid CM963 plus SR155) microaerobically for 24 h at 42°C. Then, it was streaked on CCDA and incubated microaerobically at 42°C for 24 h and examined for the presence of *Campylobacter*. To exclude the possibility of infection from another source, the isolates were Penner serotyped as described by Jacobs-Reitsma et al. (1995a).

Quantification of transmission

A susceptible-infectious (*SI*)-type model was used to describe the dynamics of transmission with time (Anderson et al., 1992; Diekmann et al., 2000). In such a model all individuals are considered to be identical and each individual can be in one of two stages: susceptible or infectious. The model then describes the changes in the fraction of individuals in the two states, with $s(t)$ being the proportion of susceptible birds at time t and $i(t)$ the proportion of infectious birds. The SI-model assumes that once a bird becomes infected, it will remain infectious during the

experimental period (Heres et al., 2004) and that contacts within the population are random. In addition, both classes S and I are assumed to be homogeneous, and the transmission rate is taken to be constant during the entire infectious period and equal for all infectious broilers.
Susceptible birds are assumed to become infected at the rate of $\beta s(t)i(t)$. The transmission rate parameter β can be defined as the average number of secondary cases caused by one infectious bird per time unit in a susceptible population (Diekmann et al., 2000). Although transmission between individuals is inherently a chance process, the dynamics in a large enough population can be approximated by a deterministic differential equation. In the case of the *SI* model we have the following equation:

$$\frac{di(t)}{dt} = \beta s(t) i(t),$$

of which the solution is the logistic curve

$$i(t) = \frac{ce^{\beta t}}{1 + ce^{\beta t}},$$

with $c = i(0)/[1 - i(0)]$, $i(0)$ being the proportion of infectious birds at $t = 0$. The curve is shown in Figure 1.

The logistic $i(t)$-curve led to logistic regression analysis to model the change in $i(t)$ over time. A delay time (τ) was added to the model to account for a possible time shift in the start-up of the epidemic process caused by, for example, the experimental setup, the strains used, the inoculation dose, age, or stochastic effects. This resulted in the following model for the log-odds of $i(t)$:

$$\ln\left[\frac{i(t)}{1-i(t)}\right] = \ln\left(\frac{i(0)}{1-i(0)}\right) + \beta(t-\tau) = \ln\left(\frac{i(0)}{1-i(0)}\right) + \beta t + a$$

The delay time reflects the time between inoculation of the seeders and the time when the first contact birds become infectious. This delay time is different from what is usually called lag phase (Newell et al., 2003), by which is meant the minimal age of the flock at which infections are observed in the field.

The model was fitted by a standard logistic regression, with $\ln[i(0)/(1-i(0)]$ as offset, t as covariate, and a as intercept. The fit resulted in an estimate for β and for a, from which the delay τ was calculated as $\tau = -a/\beta$.

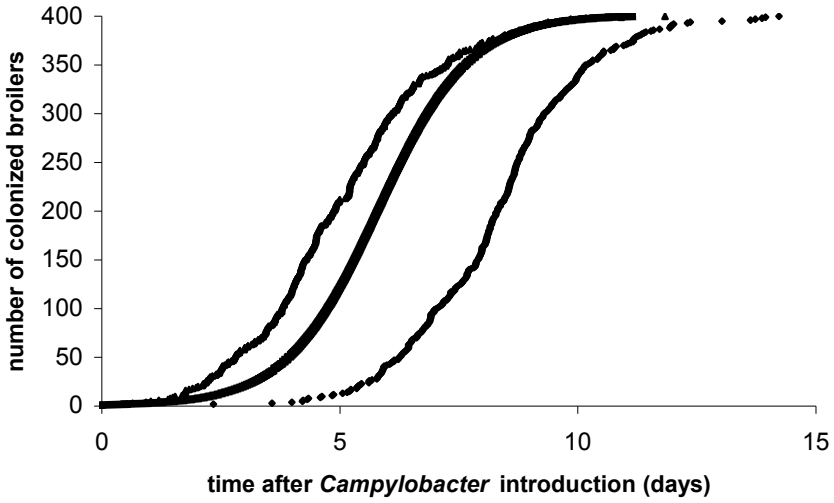

Figure. 1. Simulated course of *Campylobacter* infection in a population of 400 broilers, starting at t = 0. The middle curve is the deterministic (logistic) curve; the other two are random simulations. As can be seen, the deterministic and stochastic curves are similar, except for a time shift due to random effects in the initial phase of the outbreak.

Separate models were fitted for each experiment, resulting in four β values and four a values, and shared models were fitted in all possible combinations of these experiments, resulting in common β values for the different experiments. The Akaikes information criterion (Burnham et al., 1998) was used to decide which model had the best fit and to see whether different values of β should be adopted for different (sets of) experiments.

As an example of how β can be used, we investigated the precision with which the moment of *Campylobacter* introduction can be estimated by regularly sampling the flocks. We simulated 10,000 outbreaks in flocks of 20,000 chicks with β = 1.04 (the estimation result) and starting at time t = 0. Simulations were carried out using the so-called Sellke construction (Andersson et al., 2000): first, for each bird j a value Q_j is drawn from an exponential distribution with mean 1. Then, the epidemic is reconstructed by supposing bird j becomes infected when the cumulative infectiousness $\beta \int_0^t i(u)du$ reached Q_j, with $i(u)$ being the fraction of

infected birds at time u. In this simulation, 10, 20, or 60 birds were sampled every 1, 3, 7, or 14 days, the time of the first sample having been randomly selected from the appropriate uniform distribution, and the number of infected birds at time t was recorded. The resulting proportion of infected birds at each sampling time was then used to carry out a logistic regression analysis, as described above, in which either β was fixed at 1.04 and only a was estimated (and consequently τ), or both β and a were estimated. Because every simulation started at $t = 0$, the estimated τ is the error made in estimating the time of *Campylobacter* introduction. Thus, the 10,000 simulations yielded estimation errors for each combination of sample size (10, 20, or 60) and sampling interval (1, 3, 7, or 14 days).

RESULTS

Course of infection

Campylobacter was not detected in samples of water, feed, box liners, or wood shavings at the start of the experiments. In experiments 1 and 2, contact broilers became *Campylobacter*-positive between days 5 and 7 (Table 2).
In experiments 3 and 4, the first contact broilers became *Campylobacter*-positive between days 9 and 11 and between days 11 and 14, respectively. Pooled samples enriched in CCD broth did not become positive before the first bird was positive in the direct culture assay. In experiment 3, between days 4 and 6 more seeders were found to be positive by the enrichment method than by the direct culture method (Table 2), but this did not change the estimate of transmission.

Quantification of transmission

The logistic regression model was used to estimate β for each experiment separately and to estimate shared β values in all possible combinations. As the simplest model with a single β for all four experiments had one of the lowest Akaikes information criterion scores, there was no evidence that a more complex model was needed. The joint β was estimated at 1.04 per day with a standard error of 0.06, which means that after introduction of *Campylobacter* in a flock, each broiler will infect on average 1.04 new broilers per day. The estimated intercepts c were -2.57 (standard error [SE], 0.47), -2.77 (SE, 0.48), -6.06 (SE, 0.71), and -7.416 (SE, 0.78) for experiments 1, 2, 3, and 4, respectively. This resulted in estimated delay times of 2.4, 2.7, 5.8, and 7.1 days, respectively.

Because only four birds were initially inoculated broilers, it was hypothesized that the observed delay times, and also the differences between the estimated delay

Table 2. Number of contact infections in each experiment

Age (days)	No. of *Campylobacter*-positive broilers							
	Expt 1		Expt 2		Expt 3		Expt 4	
	Seeders [a]	Contacts [a]	Seeders	Contacts	Seeders	Contacts	Seeders	Contacts
2	0	ND[b]	0	ND				
3					0	ND	0	ND
4	3	0/50[c]	3	0/50	0[d]	0/50	2	0/50
5	4	0/50	4	0/50	1[d]	1/50	2	0/50
6					2[d]	0/50	2	0/50
7	4	9/50	3	8/50	4	0/50	2	0/50
9	4	26/50	4	25/50	4	0/50	3	0/50
11					4	1/50	3	0/50
12	4	48/50	4	45/50				
14	4	49/50	4	50/50	ND	38/50	4	20/50
16					ND	47/50	ND	40/50
18					ND	50/50	ND	49/49
23					ND	12/12	ND	12/12
28	ND	10/10	ND	10/10				
29					ND	12/12	ND	12/12
35					ND	10/10	ND	10/10
40	ND	10/10	ND	10/10				
42	ND	10/10	ND	10/10	ND	20/20	ND	20/20

[a] Number of seeders = 4; number of contacts = 50.
[b] ND, not determined.
[c] Number positive per number sampled.
[d] Enriched samples with two, three, and three positive samples in day 4, day 5, and day 6 seeders in experiment 3, respectively.

times for the four experiments, were due to change. Therefore, we tested whether the observed delay times were due to stochastic effects of the transmission process by simulating 10,000 transmission experiments with the stochastic version of the SI-model, with $\beta = 1.04$, $N = 400$, and $i(0) = 1/400$ or $1/100$. Simulations were carried out with the Sellke construction (Anderson et al., 1992) as described above. For each simulation, we determined the delay time by comparing the time it took

until $i(t) = 0.5$ with its deterministic expectation. Delay times were obtained for two different initial conditions, namely, four infected chicks, as in the experiment [$i(0) = 1/100$], and one infected chick, as an example of an extreme case of unsuccessful inoculation [$i(0) = 1/400$]. The 0.5, 2.5, 5, 50, 95, 97.5, and 99.5 delay time percentiles are shown in Table 3.

Table 3. Expected delay times under two initial conditions: one or four inoculated chicks [$I(0) = 1$ or 4][a]

$I(0)$	Expected delay time for percentile						
	0.5%	2.5%	5%	50%	95%	97.5%	99.5%
1	-1.69	-1.28	-1.09	0.36	2.89	3.57	5.13
4	-1.08	-0.82	-0.67	0.09	1.05	1.29	1.69

[a] The delay times depend on $I(0)$ and not on population size.

The observed delay times were larger than the upper percentiles of the distributions with $I(0) = 4$ (four initially inoculated chicks), which was the actual initial condition of the experiments. Only if $I(0) = 1$ could the delay times of experiments 1 and 2 be explained by chance, but this was not the case for experiments 3 and 4. Therefore, it is likely that a mechanism other than chance was responsible for the observed delay times.

Table 4 shows the 90% intervals of the estimation errors when flocks of 20,000 chicks are regularly sampled to estimate the time of *Campylobacter* introduction. If β were assumed to be 1.04, then 1-day, 3-day, and 7-day sampling intervals had comparable errors, irrespective of whether 10, 20, or 60 samples were taken. Precision decreased only with a 14-day sampling interval. If there is no information on β, then more intensive sampling would be needed, in order to generate enough data to estimate β. Note that even the most precise estimate of the time of introduction may be wrong by 3 days, due to chance effects at the beginning of the infection chain.

We also estimated β from the experiments carried out by Stern et al. (1988), who kindly provided their original data. They performed experiments with broilers aged between 4 and 42 days. We used the same *SI* model as above to estimate the

Table 4. Precision of determination of the time of *Campylobacter* introduction into a flock of 20,000 broilers, with different sample sizes and sampling intervals[a]

	90% interval of estimation error					
	β unknown[b] for sampling interval		β known (β =1.04) for sampling interval			
Sample size	1 day	3 days	1 day	3 days	7 days	14 days
10	-2.91, 4.42	-4.49, 3.97[b]	-1.13, 2.93	-1.31, 3.05	-1.72, 3.25	-3.71, 4.84
20	-2.13, 3.62	-3.04, 4.16[b]	-1.08, 2.88	-1.19, 2.94	-1.43, 3.11	-3.08, 4.34
60	-1.46, 3.09	-1.97, 3.70	-1.05, 2.87	-1.08, 2.89	-1.17, 2.91	-2.17, 3.68

[a] Denoted are the 90% intervals of the estimated introduction times of 10,000 simulated outbreaks starting on day zero.
[b] With β unknown, the introduction time could not be estimated for sampling intervals of 7 or 14 days, in only 69% of the cases with 10 samples every 3 days, and in only 90% of the cases with 20 samples every 3 days.

transmission rate. The data did not allow an estimate for each age group (too few data), but the overall estimate of β was 1.13 day^{-1} (SE, 0.04 day^{-1}), which is of the same order of magnitude as our estimate of 1.04 day^{-1}. The Stern data were more suitable than those of Shanker et al. (1990), because Stern et al. (1988) collected fecal samples more frequently at the start of the infection chain (days 3, 5, and 7).

DISCUSSION

The aim of this study was to quantify the transmission of *Campylobacter* spp. within broiler flocks. We estimated β from four experiments previously carried out by Jacobs-Reitsma et al. (1996). The estimated β value was 1.04 day^{-1}, which was comparable to the value (β = 1.3 day^{-1}) we calculated using the data of Stern et al. (2001), but it was higher than that reported by Hartnett et al. (2001) for the data of Shanker et al. (1990) (β = 0.1 to 0.3 day^{-1}). However, neither their method nor their assumptions were given explicitly. When we applied our analysis to the same data, the sampling frequency in the initial phase of colonization proved too low to allow for accurate estimation of the transmission rate.

In contrast to what is assumed to occur in the field, that is, that an infection starts with one infected bird, we started the infection with four seeders. We chose this approach to minimize the risk of an unsuccessful experiment due to a failed inoculation and the variability in the infection course, which would make it impossible to accurately quantify transmission. Starting with more than one seeder does not affect the parameter estimate, because the transmission rate is not related to the number of seeders but only depends on the number of infections and susceptible birds present. The parameter is an estimate on an individual broiler level and can be estimated properly, even when the infection starts with more than one infectious bird, but only given that a major outbreak is observed. Choosing four seeders is a compromise between guaranteeing this and staying close to a natural introduction (colonization of one bird).

Mathematical models are important tools for assessing the best means of containing an outbreak, and they help to clarify some of the associations between epidemiological factors (May et al., 1987). However, the assumptions on which a model is based should be examined carefully. The model we used assumed that the birds mixed randomly, which seems reasonable given the observations of Preston et al. (1989). However, Hartnett et al. (2001) interpreted the same data differently and assumed that broilers stay within a cluster and that clusters move. However, since our simpler mathematical model fit the experimental data well, we see no reason to introduce a more complicated model for contact structure.

The overall estimates of β did not differ significantly among experiments 1 to 4, indicating that despite various circumstances the infection processes run a similar course, but the delay time (i.e., time between inoculation of the seeders and the occurrence of the first contact infections) did differ. The delay time for experiments 1 and 2 was approximately 2 days and for experiments 3 and 4 about 5 to 7 days. Stochastic simulations with the SI-model indicated that this delay time could not be explained by chance alone. *In vitro* passage and deep frozen storage of strains might have adversely influenced the initial colonization potential of the strains (Korolik et al., 1998; Ringoir et al., 2003; Shanker et al., 1990; Stern et al., 1988; Young et al., 1999), whereas the colonization potential would be stronger after the strain had adapted to the alimentary tract of broilers, especially the seeders. This phenomenon, however, would be observed in all four experiments, as all strains were treated comparably, and so other explanations should be considered.

In experiment 1 and 2 cloacal swabs were analyzed, which might be a less sensitive method than analyzing cecal droppings, as performed in experiments 3 and 4; however, the correlation between the two methods is reported to be high (Glunder, 1995). Since the detection limits were equal for individual birds within each experimental group, this difference does not affect the shape of the epidemic curve (Fig. 1) but shifts it to the left or right. If the exact dynamics of *Campylobacter* colonization and the detection limits are known, it would be possible to correct for this, but unfortunately this was not the case. The consequence of different detection limits would be that the differences in delay time between experiments 1 and 2 and experiments 3 and 4 are even larger than reported here.

Another possible explanation for the differences in delay times between the experiments is the use of different *Campylobacter* strains, as it is known from other studies that strains differ in their colonization capacities or transmissibilities (Ahmed et al., 2002; Cawthraw et al., 1996; Jones et al., 2004; Young et al., 1999). This hypothesis might be investigated further. Also, the inoculation doses or the presence of maternal antibodies in the broilers of experiments 3 and 4 might explain the differences in delay time (Sahin et al., 2003). However, all inoculation doses were rather high, and most inoculated chicks started shedding only a few days after inoculation. Therefore, it is not likely that the dose or antibodies caused the time delay in transmission. Thus, although there are several possible explanations, the exact cause of the difference in delay times needs to be investigated further. Because the transmission rate parameter β is not affected by the delay time, this parameter can still be used in further studies to evaluate control measures for their reduction of the transmission of *Campylobacter* or to determine the within-flock prevalence over time in the field.

As with all laboratory studies, there is the question to what extent findings can be extrapolated to the field situation. However, in this instance, the problem with extrapolation is only relevant for the start of the epidemic, when the first birds become colonized and when the prevalence is still low. In this phase of the epidemic, chance processes play an important role. However, our results suggest that once the infection is spreading, the time taken to go from a prevalence of 5% to 95% will be approximately the same in various situations, allowing extrapolation to the field, which is substantiated with observations in the field (Bouwknegt et al., 2004; Evans et al., 2000). Thus, the random mixing model is robust for this phase of an epidemic, and there is no need to use a model with a more complicated contact structure.

A mechanistic model has several advantages: it forces users to identify key parameters, to provide a minimal set of mechanisms necessary to explain the data, and to state underlying biological assumptions. It may also facilitate the generation of new hypotheses (Anderson et al., 1992; Diekmann et al., 2000; Mollison, 1995). We have shown that the mathematical model fit the data of the experiments; we pinpointed the underlying assumptions and created hypothesis for the apparent delay times. Once a suitable model is available, validation of the model for field situations can be performed. Mathematical models have been used extensively to analyze epidemics of infectious diseases (Anderson et al., 1992; de Jong, 1995; Diekmann et al., 2000; Koopman, 2004; May et al., 1987; Mollison, 1995; Stegeman et al., 1999). The information they generate provides insight into the course of epidemics and can be used in attempts to reduce the incidence of various infectious diseases. Especially in the case of fastspreading infections like *Campylobacter*, gathering quantitative information on field infections can be a helpful tool in the evaluation of interventional measures. Knowledge of epidemiological mechanisms and parameters underlying *Campylobacter* transmission in broiler flocks is important for the evaluation and development of control strategies, because it enables us to determine which measures can reduce transmission and whether the magnitude of the effect is sufficient to reduce transmission or to postpone introduction, which in turn decreases the prevalence in a flock and subsequently the exposure of humans to *Campylobacter* via contaminated poultry products.

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Chapter 3

Quantifying transmission of *Campylobacter jejuni* in commercial broiler flocks

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ABSTRACT

Since meat from poultry colonized with *Campylobacter* spp. is a major cause of bacterial gastroenteritis, human exposure should be reduced by, among other things, prevention of colonization of broiler flocks. To obtain more insight into possible sources of introduction of *Campylobacter* into broiler flocks, it is essential to estimate the moment that the first bird in a flock is colonized. If the rate of transmission within a flock were known, such an estimate could be determined from the change in the prevalence of colonized birds in a flock over time. The aim of this study was to determine the rate of transmission of *Campylobacter* using field data gathered for 5 years for Australian broiler flocks. We used unique sampling data for 42 *Campylobacter jejuni*-colonized flocks and estimated the transmission rate, which is defined as the number of secondary infections caused by one colonized bird per day. The estimate was 2.37 ± 0.295 infections per infectious bird per day, which implies that in our study population colonized flocks consisting of 20,000 broilers would have an increase in within-flock prevalence to 95% within 4.4 to 7.2 days after colonization of the first broiler. Using Bayesian analysis, the moment of colonization of the first bird in a flock was estimated to be from 21 days of age onward in all flocks in the study. This study provides an important quantitative estimate of the rate of transmission of *Campylobacter* in broiler flocks, which could be helpful in future studies on the epidemiology of *Campylobacter* in the field.

INTRODUCTION

Campylobacter spp. are a common cause of diarrhea in humans, and many cases of campylobacteriosis are associated with the handling and consumption of contaminated poultry meat (Friedman et al., 2004). Studies of the epidemiology of *Campylobacter* have resulted in the implementation of biosecurity and hygienic measures on poultry farms and slaughterhouses with the ultimate goal of reducing human exposure (Gellynck et al., 2008; van de Giessen et al., 1998). These measures likely contributed to a reduction in the number of *Campylobacter*-positive broiler flocks. Nevertheless, contaminated meat is still on the market (Hansson et al., 2007), and a further reduction in the prevalence of *Campylobacter*-positive flocks is considered necessary by public health authorities in many countries (EFSA, 2005).

Clearly, more knowledge concerning the mechanism of introduction of *Campylobacter* into a flock is essential for improving the current control programs. This, in turn, requires an estimate of the moment that a flock becomes colonized. However, it does not seem feasible to detect the first bird that is colonized in a commercial broiler flock because of flock size and the necessary sampling frequency.

An alternative approach is to determine the transmission rate (β) of *Campylobacter* within a flock. β, which is defined as the number of secondary infections caused by one colonized bird per day, determines the rate of increase in the number of colonized birds over time. It can be used to determine the moment of introduction from field data on increasing *Campylobacter* prevalence over time. The estimates for β that have been obtained in experimental studies (Stern et al., 2001; van Gerwe et al., 2005) are 1.04 to 1.13 per day. However, experimental conditions differ substantially from the field situation, which implies that the β in commercial flocks should also be estimated.

A series of field studies in Australia were carried out between 1999 and 2004 in which broiler flocks were sampled daily to weekly. The aim of these studies was to develop an understanding of the epidemiology of *Campylobacter* in Australian broiler flocks. We analyzed data from the unique data set obtained in these studies to estimate the rate of *Campylobacter* transmission in commercial broiler flocks. Additionally, we estimated the moment that the first bird in a flock was colonized with *Campylobacter* (for reasons of convenience, we refer to this event as the

moment of introduction of *Campylobacter* into a flock) and assessed how accurately moments of introduction can be estimated.

MATERIALS AND METHODS

Data set

Three longitudinal studies were carried out in Southeast Queensland, Australia, between 1999 and 2004. A subset of the full data set was selected for this study; for flocks to be included in this study, they had to have been sampled at least twice, and at least one *Campylobacter*-positive dropping had to be detected. Flocks with migration barriers separating groups of birds within a shed and flocks whose sampling age was not stated were excluded. A total of 42 flocks met all the inclusion criteria (see the supplemental material for details).

At each sampling age randomly selected individual fecal or cecal droppings were collected immediately after the droppings were produced. Birds that produced these droppings were not marked, as the likelihood of sampling a single bird more than once a day was considered limited for the large populations used. Care was taken to ensure that each sample was collected without any contaminating material. The samples were collected with a sterile swab, which was immediately placed in a sterile container. Samples were kept on ice during transport to the laboratory and were streaked on Karmali *Campylobacter* agar base (Oxoid CM935; Oxoid, Melbourne, Australia) containing *Campylobacter* selective supplement (Oxoid SR167E) immediately after arrival in the laboratory. Agar plates were incubated at 42°C for 48 h in an incubator with an atmosphere consisting of 85% N_2, 10% CO_2, and 5% O_2. Colony morphology and cell motility as determined by phase-contrast microscopy were used to confirm identification to the genus level. Single colonies from a number of positive samples from each positive flock were subcultured on sheep blood agar and incubated as described above before they were identified to the species level by oxidase, catalase production, and hippurate hydrolysis tests.

The final data set consisted of data for sampling events for each flock, included the age of the flock (t), sample size (n_t), flock size at the start of the rearing period (N), and number of positive samples (x_t) (see the supplemental material for details).

Modeling of *Campylobacter* transmission in broiler flocks

It is generally assumed that following the onset of *Campylobacter* colonization of the gut, broilers shed these bacteria for the rest of their lives (Achen et al., 1998).

Therefore, we assumed that birds were either susceptible (noncolonized) or infectious (colonized) and that an increase in prevalence can be described by a susceptible-infectious (SI) type of mathematical model (Keeling et al., 2008; van Gerwe et al., 2005). In this model, susceptible chicks can be colonized upon contact with an infectious chick, which occurs at rate $\beta i(t)$, where $i(t)$ is the proportion of infectious chicks in the shed. β is considered the mean number of chickens that can be colonized by one infectious chicken per day in a susceptible population.

In the large populations of broilers usually present in commercial flocks, the change in $i(t)$ can be approximated by the deterministic differential equation:

$$\frac{di(t)}{dt} = \beta(1 - i(t))i(t) \tag{1}$$

whose the solution is the logistic curve described by

$$i(t) = \frac{e^{\beta(t-\tau)}}{1 + e^{\beta(t-\tau)}} \tag{2}$$

in which τ is the time at which 50% of the birds are infected. From τ, β and N, the time when transmission starts (t_0) is calculated as follows:

$$t_0 = \tau - \log(N-1)/\beta \tag{3}$$

Estimation of β in the study population

Because the increase in $i(t)$ follows a logistic curve, β is estimated by a logistic regression analysis of x_t. However, this is possible only if for at least two samples only some of the swabs are positive, because only then is the steepness of the increase in prevalence (determined by β) observed. Eight flocks met this criterion. The SI-model was fitted for each of these eight flocks by logistic regression of the binomially distributed x_t (and n_t) with t as covariate and exp(τ) as the intercept. The model fits resulted in eight estimates for β and τ, from which t_0 could be estimated by using equation 3. Confidence intervals for β and t_0 were derived by profile likelihood analysis (Dohoo et al., 2003), which was carried out using Mathematica (version 6.0; Wolfram Research, Inc. [http://www.wolfram.com]).

Because flocks with low β have been more likely to meet the inclusion criteria for the logistic regression analysis than flocks with high β, bias would be introduced if

the eight estimates were considered representative of all commercial broiler flocks. Therefore, in the next step, we estimated the mean (μ_β) and standard deviation (σ_β) of a normal distribution of β among all flocks by maximizing the likelihood function

$$\ell(\mu_\beta, \sigma_\beta) = \prod_{flocks}\left[\int_0^\infty p(\beta)\prod_t i(t)^{x_t}\left(1-i(t)\right)^{n_t-x_t} d\beta\right] \quad (4)$$

Where $p(\beta)$ is the density of the normal distribution of β, $i(t)$ is the logistic curve for equation 2, and t, x_t, and n_t are the data for all flocks. This maximum likelihood estimation method does not result in any flock-specific estimates for β or t_0; estimation results are limited to μ_β and σ_β, with 95% confidence intervals derived by profile likelihood (Dohoo et al., 2003). Although β cannot be negative, the assumption of a normal distribution is valid, because the distribution is well above zero (see Results). The analysis was carried out using Mathematica.

Estimation of the t_0 for the study population

Because no flock-specific estimates of the moment of introduction (t_0) could be obtained with the analysis described above, we used Markov chain Monte Carlo integration to obtain Bayesian posterior distributions of β and τ, and therefore t_0, for each flock separately (Gelman et al., 2003). The prior distribution of τ was uninformative (flat), whereas the prior distribution of β was the normal distribution resulting from maximum likelihood estimation (with $\mu_\beta = 2.37$ day^{-1} and $\sigma_\beta = 0.295$ day^{-1}). The likelihood function was

$$\ell(\beta, \tau) = \prod_t i(t)^{x_t}\left(1-i(t)\right)^{n_t-x_t} \quad (5)$$

We used the maximum likelihood results for all 42 flocks as input for estimation of t_0 in the same flocks, but it would have been more correct to divide the data into two mutually exclusive subsets and use each subset only once. However, the approach which we used resulted in more precise estimates of μ_β and σ_β and estimates of t_0 for all flocks instead of only a subset. The possible errors resulting from our approach were minor, as determined by a separate sensitivity analysis of estimation of t_0 to correctness of the prior distribution of β (see below).

The posterior distribution was sampled 10,000 times by single-component Metropolis-Hastings sampling, after 100 samples were used for burn-in (Gilks et al., 1996). The means of the (normal) proposal distributions for the (i + 1)th samples of both β and τ were the ith samples. The standard deviation of the

proposal distribution of β was 0.295 day $^{-1}$; the standard deviation of the proposal for τ was determined from the data set, and it was one-quarter of the time interval between the last 0% prevalence sample and first 100% prevalence sample. This resulted in means and 95% credible intervals for t_0 for 40 of the 42 flocks (for 2 flocks, the flock size was not known). The sampling algorithm was programmed using Mathematica.

Accuracy of t_0 estimation

Although Bayesian estimation of t_0 does provide 95% credible intervals, these are based on a logistic curve which does not take account of the stochastic nature of transmission in the early phase of a *Campylobacter* outbreak. Therefore, we assessed the accuracy of the method by estimating t_0 using simulated outbreaks in which the estimates could be compared to the real value.

We simulated 10,000 outbreaks in flocks consisting of 20,000 chicks with values for β sampled from the estimated distribution (μ_β = 2.37 day^{-1}; σ_β = 0.295 day^{-1}), with a t_0 of 0. Simulations were carried out as described previously (van Gerwe et al., 2005) with three sample sizes (10, 20, and 100 birds) and three sampling frequencies (once every day, every third day, or every seventh day). This resulted in 10,000 simulated data sets for all combinations of sample sizes and sample frequencies, which were subsequently analyzed by the Bayesian method described above to obtain posterior means for 1,000 samples of the posterior distribution.

In addition to these 10,000 simulations, where the population distribution of β (used in the simulations) was identical to the prior distribution in the Bayesian analysis, we assessed the accuracy by simulation with other distributions, based on the confidence intervals for μ_β (2.19 to 2.58 day^{-1}) and σ_β (0.144 to 0.488 day^{-1}). Four new data sets were simulated, including two data sets with different μ_β (2.19 and 2.58 day^{-1}, with σ_β kept at 0.295 day^{-1}) and two data sets with different σ_β (0.144 and 0.488 day^{-1}, with μ_β kept at 2.37 day^{-1}). This resulted in four sets of 1,000 simulated data sets for 10 samples every 1, 3, or 7 days. These data were used to estimate t_0 as described above.

RESULTS

Data set

The flock age at the time of first detection of *Campylobacter*-positive droppings varied from 24 to 54 days. For 14 of 42 flocks the prevalence was more than 95%

at the time of first detection. For an additional 10 flocks in which the prevalence was up to 10% at the time of first detection, sampling was continued until the flocks were found to be fully colonized (>95% prevalence), which took 4.4 days on average (range, 3 to 6 days) (see the supplemental material for details).

For 38 of 42 flocks, all *Campylobacter* isolates were identified as *Campylobacter jejuni*. Both *C. jejuni* and *Campylobacter coli* were found in four flocks. Because insufficient data concerning *C. coli* outbreaks were available, our analyses were based solely on *C. jejuni* outbreaks.

Estimation of β and t_0 for the study population. For eight flocks with sufficient data, flock-specific β and t_0 were estimated by logistic regression. The point estimates for β ranged from 1.3 to 3.1 day^{-1} (Figure 1), and t_0 ranged from 21 to 35 days (see the supplemental material for details).

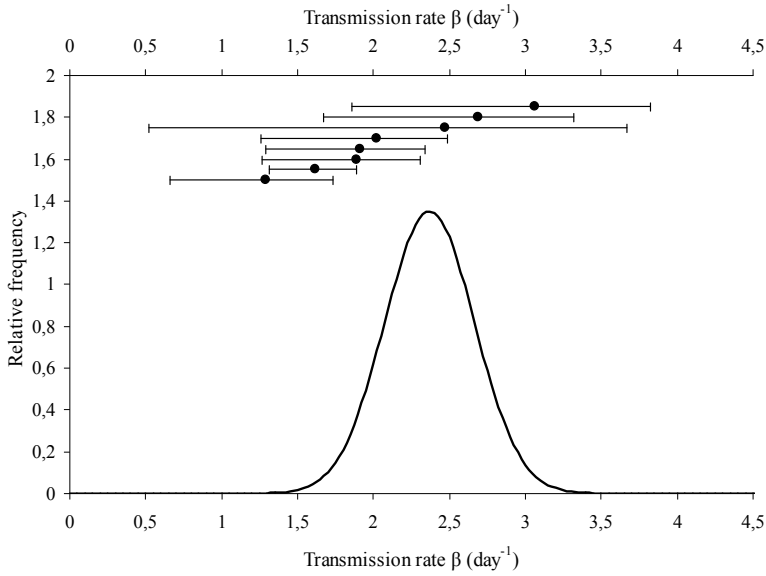

Figure 1. (Top): Eight estimates of *Campylobacter* β (with 95% confidence intervals), obtained by logistic regression. (Bottom) Estimated density of the normal distribution of β in the Australian study population, obtained by maximum likelihood estimation (μ_β = 2.37 day^{-1}; σ_β = 0.295 day$^{-1)}$.

The estimates for the distribution of β, which were based on 42 outbreaks, were as follows: μ_β = 2.37 day^{-1} (95% confidence interval, 2.19 to 2.58 day^{-1}) and

$\sigma_\beta = 0.295$ (95% confidence interval, 0.144 to 0.488 day^{-1}) (Figure 1). Figure 2 shows how fast flocks of 20,000 broilers were colonized for the mean β, a low β, and a high β within the distribution of β.

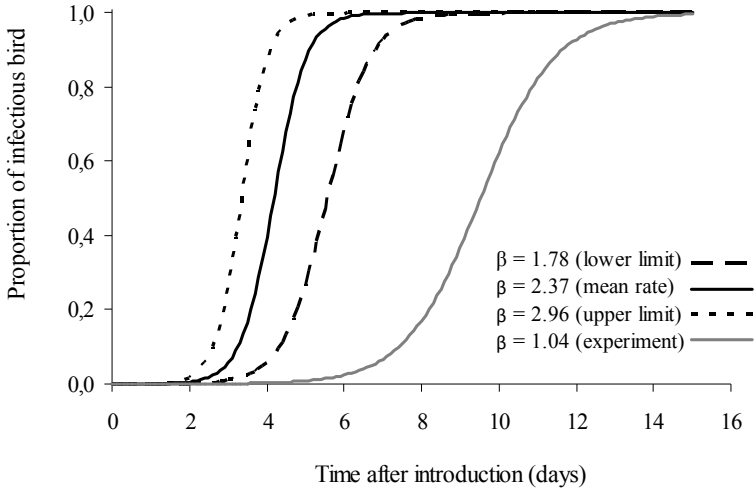

Figure 2. Epidemic curves for three different values of the estimated distribution of β (lower limit, point estimate, and upper limit) and an experimentally derived β estimate (van Gerwe et al., 2005) for flocks consisting of 20,000 broilers.

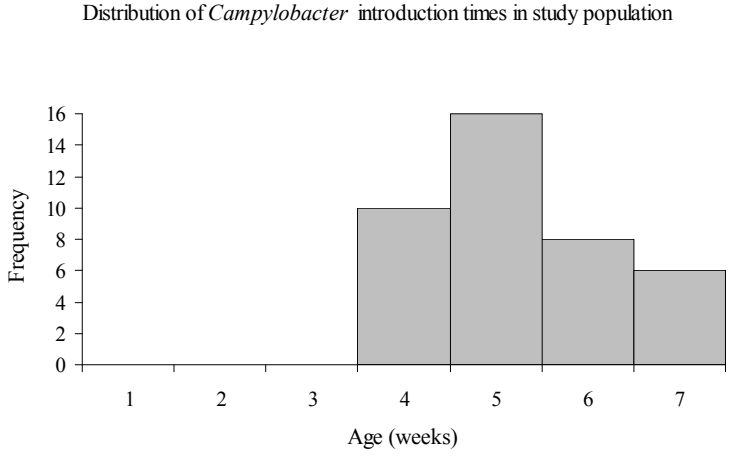

Figure 3. Histogram showing the t_0 for 40 Australian *C. jejuni* outbreaks.

For 40 *Campylobacter* outbreaks in which the population size was known we estimated t_0 by Bayesian analysis. The results (Figure 3) show that colonization did not take place before chickens reached an age of 3 weeks. On average, t_0 was 4.8 days (range, 2.2 to 9.3 days) earlier than the first detection of *Campylobacter*-positive samples (see the supplemental material for details).
The estimates obtained by logistic regression and Bayesian analysis did not differ more than 0.6 day except for one flock (3.4-day difference).

Accuracy of t_0 estimation
The accuracy of the Bayesian method was assessed by analyzing simulated outbreaks; the results are shown in Table 1. If the prior distribution used for the analysis reflected the underlying β in the population correctly, the t_0 was estimated within 3 days even with weekly sampling of only 10 birds.

Table 1. Accuracy of Bayesian estimation (with prior distribution $\mu_\beta = 2.37$ day^{-1} and $\sigma_\beta = 0.295$ day^{-1}) of the posterior mean time of *Campylobacter* introduction into a flock of 20,000 broilers, with different sample sizes and sampling intervals.

				90% Interval of estimation error		
μ_β (day^{-1})	σ_β (day^{-1})	No. of data sets	Sample size	Each day	Each 3rd day	Each 7th day
2.37	0.295	10,000	10	-0.9 to 1.7	-1.0 to 1.8	-2.1 to 2.7
		10,000	20	-0.8 to 1.6	-0.9 to 1.7	-1.8 to 2.4
		10,000	100	-0.6 to 1.4	-0.8 to 1.6	-1.3 to 2.1
2.19	0.295	1,000	10	-0.7 to 2.2	-0.8 to 2.4	-1.7 to 3.1
2.58	0.295	1,000	10	-1.1 to 1.3	-1.4 to 1.3	-2.5 to 2.3
2.37	0.144	1,000	10	-0.8 to 1.4	-0.8 to 1.4	-1.9 to 2.5
2.37	0.488	1,000	10	-1.0 to 2.3	-1.3 to 2.7	-2.4 to 3.3

The Bayesian estimation method was not very sensitive to the correctness of the prior distribution (within the margins of the 95% confidence intervals), and the

sensitivity was greatest if μ_β was lower or σ_β was higher, resulting in a margin of 3.3 days with weekly sampling of 10 birds.

DISCUSSION AND CONCLUSION

The first aim of this study was to determine the β of *Campylobacter* in commercial broiler flocks. β, based on the field data, was estimated to be 2.37 ± 0.295 day^{-1}, which means that one colonized bird could, on average, infect 2.37 birds per day. This implies that in a flock of 20,000 broilers the within-flock prevalence of *Campylobacter* would increase from a prevalence of one colonized bird to a prevalence of 95% within 1 week.

Previous studies showed that flocks determined to be *Campylobacter* negative at one moment could appear to be fully colonized within 1 week, suggesting that *Campylobacter* was introduced in the intervening week (Allen et al., 2008; Hald et al., 2001; Jacobs-Reitsma et al., 1995). This seems consistent with our findings, but the sample size used in these studies did not allow detection of positive flocks at the start of an epidemic. This implies that bacteria could have been introduced at some previous time.

The second aim of this study was to estimate the moment of colonization of the first bird in a flock (t_0) by using the estimated β. The estimates of t_0 were all greater than a flock age of 21 days. The estimate of t_0 was accurate within 3 days in 90% of the cases, even if only 10 birds were sampled weekly. The sensitivity to incorrectness of the prior distribution was low. The apparent lack of *Campylobacter* transmission in the first 3 weeks of life, which seemed to have occurred in our study, has been described previously (Jacobs-Reitsma et al., 1995). It is difficult or probably impossible to determine whether in the first 3 weeks no introduction occurred or whether introduction of bacteria did occur but did not result in colonization of the birds. The risk of introduction of *Campylobacter* can be high throughout the rearing period, but it could also be hypothesized that this risk may increase over time. Another possibility is that young chicks are less susceptible, as indicated by Ringoir et al. (2007) and Sahin et al. (2003), who demonstrated that age and maternally derived immunity had an effect on susceptibility. More insight into the underlying mechanism of this phenomenon may provide clues for prevention of *Campylobacter* colonization.

A unique data set describing the change in prevalence of *Campylobacter*-colonized birds in commercial broiler flocks was used to quantify *C. jejuni* transmission under field conditions. The estimated β implies that in a flock of 20,000 broilers the within-flock prevalence of *Campylobacter* increases to 95% within 1 week after colonization of the first bird. Since such rapid spread has not been described previously on the basis of experimental transmission experiments, this study provides important new quantitative information on the epidemiology of *Campylobacter* in broilers.

This study also showed how the β can be used to estimate when the first bird is colonized. We showed that this method for estimation was accurate, and therefore this method is a promising method for further studies of mechanisms of *Campylobacter* introduction, because it allows focusing on the chronology of events.

Additionally, t_0 estimation can result in an accurate description of the period in which no transmission of *Campylobacter* occurs, like the first 3 weeks for our study population. Consequently, interventions aimed at prevention of introduction of and subsequent colonization by *Campylobacter* might better be targeted at the second half of the rearing period, which in our study population could be considered a high-risk period.

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Supplemental material

Quantifying Transmission of *Campylobacter jejuni* in Commercial Broiler Flocks

by

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Records of sampling events for each of 42 flocks, used for estimation of the transmission rate of *Camplylobacter jejuni* in commercial broiler flocks, with flock size at the start of the rearing period N, age of the flock t, sample size n_t, number of positive samples x_t, and results (Bayesian estimate value for t_0 for 40 flocks, with logistic regression estimates of t_0 and b for 8 flocks) with 95% confidence intervals between brackets.

flock	N	t	n_t	x_t	results
1	NA[1]	7	10	0	NA[1]
		14	20	0	
		21	20	0	
		28	20	0	
		35	20	2	
2	36.000	0	50	0	27.1(25.2-29.2)
		7	100	0	
		14	100	0	
		21	100	0	
		28	100	0	
		35	100	100	
3	NA	7	10	0	NA
		14	20	0	
		21	20	0	
		28	20	0	
		35	20	1	
4	20.400	21	100	0	26.3(25.2-27.2)
		28	100	0	
		31	100	78	
		34	30	30	
5	20.300	21	100	0	25.4(24.2-26.4)
		28	100	2	log regression:
		31	100	86	t_0 24.8(23.2-26.3)
		34	30	30	β 1.91(1.48-2.53)
6	19.430	0	25	0	28.4(26.1-30.1)
		7	100	0	
		14	100	0	
		21	100	0	
		28	100	0	
		35	100	99	
		42	30	30	
7	23.500	0	22	0	34.5(32.5-36.7)
		7	100	0	
		14	100	0	
		21	100	0	
		28	100	0	
		35	100	0	
		42	30	30	
8	19.045	14	10	0	31.8(30.3-33)
		21	10	0	
		28	10	0	
		31	10	0	
		34	10	0	
		37	10	9	
9	19.229	14	10	0	32.1(30.5-33.3)
		21	10	0	
		28	10	0	
		31	10	0	
		34	10	0	
		37	10	8	

flock	N	t	n_t	x_t	results
10	17.650	14	10	0	30.1(28.7-31.2)
		21	10	0	
		28	10	0	
		31	10	0	
		34	10	3	
		37	10	10	
11	17.992	14	10	0	27.8(26.5-29)
		21	10	0	
		28	10	0	
		31	10	1	
		34	10	10	
		37	10	10	
12	22.845	0	50	0	
		7	100	0	
		14	100	0	28.4(27.2-29.4)
		21	100	0	log regression:
		28	100	0	t_0 27.8(26.1-29.3)
		31	100	2	β 1.89(1.47-2.51)
		34	100	85	
		37	100	100	
		42	30	30	
13	23.536	14	10	0	31.3(29.9-32.6)
		21	10	0	
		28	10	0	
		31	10	0	
		34	10	0	
		37	10	10	
14	19.440	14	10	0	
		21	10	0	
		28	10	0	
		29	10	0	
		30	10	0	
		31	10	0	
		32	10	0	
		33	10	0	37.1(35.9-38)
		34	10	0	log regression:
		35	10	0	t_0 37.3(33.7-39.1)
		36	10	0	β 2.47(1.27-4.42)
		37	10	0	
		38	10	0	
		39	10	0	
		40	10	0	
		41	10	4	
		42	10	8	
		46	10	10	

[1]NA = Not Available

flock	N	t	n_t	x_t	results
15	19.920	14	10	0	
		21	10	0	
		28	10	0	
		29	10	0	
		30	10	0	30.5(28.8-31.8)
		31	10	0	log regression:
		32	10	0	t_0 27.1(23.1-29.7)
		33	10	1	β 1.29(0.85-1.92)
		34	10	0	
		35	10	2	
		36	10	7	
		37	10	8	
		38	10	9	
		39	10	10	
16	18.602	14	10	0	39.9(38.4-41.5)
		21	10	0	
		28	10	0	
		29	10	0	
		30	10	0	
		31	10	0	
		32	10	0	
		33	10	0	
		34	10	0	
		35	10	0	
		36	10	0	
		37	10	0	
		38	10	0	
		39	10	0	
		40	10	0	
		41	10	0	
		42	10	0	
		46	10	10	
17	23.737	0	50	0	
		7	100	0	
		14	100	0	
		21	100	0	
		28	100	0	
		29	100	0	
		30	100	0	
		31	100	0	
		32	100	0	35.6(34.6-36.4)
		33	100	0	log regression:
		34	100	0	t_0 35.1(33.9-36)
		35	100	0	β 1.62(1.35-1.93)
		36	100	0	
		37	100	0	
		38	100	0	
		39	100	0	
		40	100	1	
		41	100	53	
		42	100	79	
		46	100	96	
		52	30	30	

flock	N	t	n_t	x_t	results
18	24.100	14	10	0	47.8(46.3-48.8)
		21	10	0	
		28	10	0	
		29	10	0	
		30	10	0	
		31	10	0	
		32	10	0	
		33	10	0	
		34	10	0	
		35	10	0	
		36	10	0	
		37	10	0	
		38	10	0	
		39	10	0	
		40	10	0	
		41	10	0	
		42	10	0	
		46	10	0	
		52	10	4	
19	25.900	29	100	0	37.9(36-39.3)
		35	100	0	
		44	100	97	
20	24.400	29	100	0	35.2(32.6-38.2)
		35	100	0	
		44	100	100	
21	24.300	29	100	0	37.7(35.5-39.3)
		35	100	0	
		44	100	98	
22	24.000	29	100	0	44.7(41.6-48)
		35	100	0	
		44	100	0	
		54	100	100	
23	17.000	27.5	100	0	44.4(43-45.4)
		37.5	100	0	
		45.5	100	0	
		49.5	100	91	
24	22.000	27.5	100	0	37.3(35-39.8)
		37.5	100	0	
		45.5	100	100	
25	20.000	27.5	100	0	41.1(39.7-42)
		37.5	100	0	
		45.5	100	57	
		49.5	30	30	
26	20.000	27.5	100	0	43.8(42.7-44.8)
		37.5	100	0	
		45.5	100	0	
		49.5	100	99	
27	20.000	27.5	100	0	43.7(42.7-44.6)
		37.5	100	0	
		45.5	100	0	
		49.5	100	100	
28	20.000	27.5	100	0	42.5(41.5-43.2)
		37.5	100	0	
		45.5	100	6	
		49.5	30	30	

flock	N	t	n_t	x_t	results
29	20.000	24	100	3	21.2(20.3-22)
		28	100	99	log regression:
					t_0 20.8(19.4-22)
					β 2.02(1.55-2.78)
30	19.000	28	100	0	31.2(29.5-33)
		32	100	0	
		38	30	30	
31	19.000	28	100	0	24.7(22-24.4)
		35	100	0	
		38	100	10	
32	18.000	28	100	0	33.8(32.6-34.8)
		35	100	0	
		38	100	50	
33	19.000	28	100	0	32.5(31.8-33.1)
		35	100	3	
		38	100	100	
34	20.000	28	100	67	23.4(22-24.4)
		35	30	30	
35	21.977	28	100	0	30.5(28.9-31.7)
		36	100	92	

flock	N	t	n_t	x_t	results
36	21.908	28	100	0	30.2(28.3-31.5)
		36	100	96	
37	22.537	28	100	0	28.8(27.3-30)
		34	100	86	
38	15.500	24	100	0	26.4(25.3-27.2)
		27	100	0	
		30	100	22	
39	18.500	28	100	1	25.8(25-26.5)
		31	100	99	log regression:
					t_0 26.3(25.2-27.4)
					β 3.06(2.3-4.26)
40	18.500	26	100	0	28.2(27.1-29)
		29	100	0	
		32	100	26	
41	20.000	24	100	0	24.7(23.9-25.5)
		27	100	1	log regression:
		30	100	97	t_0 25(23.8-26.3)
					β 2.69(2.06-3.71)
42	19.000	24	100	0	27.4(26.1-28.8)
		27	30	0	
		30	30	1	

Chapter 4

Medium chain fatty acid feed supplementation reduces the probability of *Campylobacter jejuni* colonization in broilers

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Submitted

ABSTRACT

Campylobacteriosis in humans is associated with handling and consumption of contaminated broiler meat. Reduction of the number of *Campylobacter* colonized broiler flocks could potentially be realized by decreasing their susceptibility for colonization. The aim of this study was to determine the effect of feed supplementation with a mixture of medium chain fatty acids (C_8-C_{12}) on susceptibility for *Campylobacter*, feed conversion and body weight gain. Two experiments were carried out with individually housed commercial broilers. The birds were fed with medium chain fatty acids supplemented feed (n=227), or received feed without supplement (n=87). The birds were inoculated with a dose of *Campylobacter jejuni* varying between 1.19 - 5.47 ^{10}Log cfu. During 14 days after inoculation, cecal or fecal samples were collected repeatedly, in which the presence of *Campylobacter* was determined by bacterial culture. Beta-binomial dose response modeling of the colonization status at 4 and 14 days post-inoculation was performed to estimate the *C. jejuni* dose necessary to colonize 50% of inoculated broilers, which was estimated to be 200 times higher in broilers fed with supplemented feed (4.8 ^{10}Log cfu) than in control broilers (2.5 ^{10}Log cfu). Feed conversion was not affected by feed supplementation, while body weight gain was 49 g higher in broilers fed with supplemented feed. These findings indicate that susceptibility of broilers for *Campylobacter* colonization is decreased by supplementation with medium chain fatty acids, and that medium chain fatty acids are a promising tool for the reduction of *Campylobacter* colonization in commercial broiler flocks.

INTRODUCTION

Meat products from broilers colonized with *Campylobacter jejuni* are considered an important source of human campylobacteriosis (Friedman et al., 2004), and reduction of human exposure is an important goal of public health programs (EFSA, 2005). One way to achieve this is to prevent intestinal colonization of broilers with *Campylobacter* spp., by either prevention of exposure to the bacteria or by reducing the susceptibility for *Campylobacter* colonization of broilers (Wagenaar et al., 2006). Biosecurity measures aimed to prevent introduction of the bacteria might be difficult to implement at a highly effective level. Reduction of the susceptibility, here defined as the probability of colonization upon exposure, may be an alternative way to reduce the number of colonized flocks.

One way to reduce the susceptibility is to change the micro-environment in the gastro-intestinal tract in such a way that ingested *Campylobacter* bacteria are inactivated or unable to reach the lower intestines. It has been shown that short and medium chain fatty acids (SCFA and MCFA, respectively) have *in vitro* anti-*Campylobacter* activity that is additional to the *Campylobacter* inactivating effect of an acidified micro-environment (Chaveerach et al., 2002; Thormar et al., 2006). Feed supplemented with these fatty acids might therefore result in inactivation of *Campylobacter* both by their own anti-*Campylobacter* activity and by acidification of the digesta, resulting in a reduced chance of colonization in broilers.

The effect of SCFA-supplemented feed on *Campylobacter* colonization has been studied, using high concentrations of these acids (Heres et al., 2004). Indeed colonization was reduced, but body weight gain (BWG) was also reduced, which was most likely caused by reduced feed intake. As BWG is an economically important determinant for the profitability in the commercial broiler industry, farmers are most likely reluctant to implement this particular intervention. This implies that the composition of the feed supplement should be adapted in order to improve feed intake and technical performance.

Lower concentrations of fatty acids might also reduce susceptibility, especially when fatty acids that have anti-*Campylobacter* activity throughout a larger part of the gastro-intestinal tract are used. The anti-*Campylobacter* activity of SCFA in buffered solutions ceases at pH ≥5.5 (Chaveerach et al., 2002). It has been shown that 1-Monoglycerid of Capric acid, a MCFA, had anti-*Campylobacter* activity in feed mixed with a buffer at pH 5.5 and in feed mixed with tap water at pH 7.0 (Thormar et al., 2006). Consequently, anti-*Campylobacter* activity of SCFA will

most probably be limited to the crop (pH 4.5), the proventriculus (pH 4.4), and the gizzard (pH 2.6) (Durant et al., 1999; Farner, 1942), whereas MCFA might also inactivate *Campylobacter* cells in the intestines, where the pH is approximately 5.8-6.0 (Chang and Chen, 2000; Farner, 1942). A low concentration mixture of MCFA could therefore be a promising tool to decrease the susceptibility without negative effects on weight gain or feed conversion.

The aim of this study was to determine whether an acidified feed containing 1% MCFA was able to reduce the susceptibility of broilers for colonization with *Campylobacter jejuni*. The effect of MCFA feed supplementation on the susceptibility of broilers was assessed experimentally by inoculation of broilers with various doses of *C. jejuni*. To quantify the effect on susceptibility, the inoculation dose that colonizes 50% of inoculated broilers (CD_{50}) (Line et al., 2008) was estimated for both treatments: broilers provided supplemented feed (SF) and broilers provided control feed (CF). Additionally, the effects of supplemented feed on BWG and feed conversion rate (FCR) were determined.

MATERIALS AND METHODS

Experimental Design

Two experiments were carried out subsequently. For each trial hatching eggs, originating from a commercial broiler breeder flock (Ross 308) were purchased at 17 days of incubation. Chicks were hatched at the experimental facilities of the Faculty of Veterinary Medicine of Utrecht University. After hatching broilers were randomly divided in two groups, the control (CF) and the treatment (SF) group, and received the corresponding type of feed throughout the experiments. CF was an antibiotic and anti-coccidia drug free, mashed diet (main components wheat (36.5%), soy (21.2%), corn (20%), peas (15%), and soy-oil (3.95%)). SF was the same diet, with 1% soy-oil substituted by 1% Lodestar^{TM} C8-10 (Loders Croklaan, Wormerveer, the Netherlands). This product is produced by fractional distillation of palm kernel oil free fatty acids, and typically consists of 56% C_{10}, 30% C_8, 10% C_{12}, <3% C_6, and <3% other lipids.

In experiment 1 (exp. 1), at day of hatch, a group of 150 chicks was provided CF, and a group of 47 chicks was provided SF. In this experiment, diarrhea, depression and growth retardation were observed in the group of SF broilers around 1 week of age. Because these symptoms could have been feed related, in experiment 2 (exp. 2), at day of hatch, 192 day-old chicks were randomly divided into 32 groups of 6

chicks each, 22 groups receiving CF and 10 groups SF. Thus we could test for a relation between feed and any of the above symptoms, would they occur. No such relation could be established. Birds were housed in a well-controlled facility, on litter floors. Groups were separated by walls. Water and feed was available *ad libitum*, and from 7 days of age a daily dark period of 6 hours was applied.

At 14 days of age, 114 CF and 42 SF broilers (exp. 1), and 113 CF and 43 SF broilers (exp. 2) were randomly selected, weighed and housed individually in wired cages of 40x40 cm, with closed, littered floors, which were situated in four identical compartments. The sides and back of each cage were covered with plastic sheets. The distance between the cages was 40 cm at least. In each compartment, 4 randomly selected cages housed a non-inoculated broiler (sentinel) to detect *Campylobacter* transmission. In exp. 1, broilers belonging to both treatment groups were randomly divided over the cages in all compartments. In exp. 2, broilers per treatment group were evenly divided over the compartments, and within each compartment, broilers were randomly assigned to cages.

Inoculation

One dose of 10^9 cfu of *Campylobacter jejuni* C356, originating from a broiler flock (Jacobs-Reitsma et al., 1995) and stored in glycerol at –80°C, was orally administered to three 5-day-old broilers, which were housed with 3 non-inoculated contact broilers. Three days post-inoculation (PI) *C. jejuni* was isolated from the ceca of one contact broiler. This chicken-passaged strain, here referred to as *C. jejuni* C356P (C356P), was stored in glycerol at –80°C and used in experiments 1 and 2.

Before inoculation, C356P was freshly cultured in Heart Infusion Broth (micro-aerobically, 37°C, overnight) and diluted in saline to obtain the intended inoculation doses. Based on power calculations, using results from a pilot study (data not shown), and presuming an additive effect on CD_{50} of approx. 1.5 ^{10}Log, SF broilers were inoculated with higher doses C356P than CF broilers (tables 1 and 2). Broilers, except sentinels, were orally inoculated with 0.25 ml of the C356P inoculation suspensions at 14 days (exp. 1; n=140) or 18 days (exp. 2; n=139) of age, with inoculation dose randomly divided over the compartments. In exp. 2 a SF broiler died one day prior to inoculation. The concentration of *Campylobacter* in the administered inocula was determined by plating on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Biotrading Benelux B.V., Mijdrecht, the Netherlands). To stimulate feed uptake following inoculation, broilers were

feed-deprived for 11 hours prior to inoculation, and provided feed directly afterwards.

Sampling and Testing

Six (exp. 1) or 7 days (exp. 2) prior to inoculation, broilers were tested for the presence of *Campylobacter*, by culture of a fecal dropping on mCCDA. All broilers tested negative. Birds were sampled at 4, 8, and 11 days post inoculation (PI) by swabbing fresh cecal droppings if present, or otherwise a swab of a fresh fecal dropping. If neither could be obtained, a swab from cloacal content was taken. In addition, at 1, 2, 3, 7, and 9 days PI 1 sentinel per compartment and 1 to 5 inoculated broilers per dose group (see tables 1 and 2) were placed in cardboard boxes with wire floors for 4 hours, to collect cloacal swabs and fresh cecal droppings (or fecal droppings if not present). Different material was sampled with the intention to compare the sensitivity of each of these sampling methods. Because positivity was limited to one of two sample types only incidentally, sensitivity of different sample mediums was considered equal. At the end of the trial (14 days PI), cecal contents were sampled after euthanization.

The person sampling the broilers was blinded to dose groups and could not distinguish sentinels from inoculated broilers. Long-sleeved plastic gloves were changed for each broiler to avoid cross-contamination. Samples were collected with sterile swabs and transported to the laboratory in modified Amies transport medium without charcoal (Biotrading, Mijdrecht, the Netherlands) within 6 hours. Samples were streaked on mCCDA plates and incubated micro-aerobically at 42°C, and examined for the presence of *Campylobacter*-suspect colonies after 24 and 48 hours. Microscopic examination of morphology and motility was used as confirmation. Broilers were considered *Campylobacter*-colonized and were excluded from further sampling when at least one sample tested positive. Ethical aspects of the experiments were judged and approved by the animal ethical committee of Utrecht University.

Statistical Analyses

The effect of MCFA feed supplementation on susceptibility was assessed by fitting a Beta-binomial dose response model (Teunis and Havelaar, 2000). According to this model, inoculation with dose D results in a probability of colonization $\Pr_{inoc}(D)$:

$$\Pr_{inoc}(D) = 1 - \left(1 + \frac{D}{\beta}\right)^{-\alpha} \quad (1)$$

The parameters α and β are parameters of a Beta distribution of the probability of colonization per inoculated bacterium.

Because sentinels were detected positive after day 4 PI in exp. 2 (table 2), colonization as a result of transmission could not be excluded for the inoculated birds either. Therefore, two analyses were performed: the first with all data up to day 4 PI under the assumption that there was no transmission; and the second with all data up to day 14 PI, under the assumption that colonization by transmission had occurred with probability $\Pr_{tr}$, different for each room and treatment group. In the day-4 analysis, $\Pr_{+}(D)$, the probability of being colonized, was equal to $\Pr_{inoc}(D)$, as colonization was assumed to be caused by inoculation only. In the day-14 analysis, $\Pr_{+}(D)$ was equal to

$$\Pr_{+}(D) = 1 - (1 - \Pr_{tr})(1 - \Pr_{inoc}(D)) \quad (2)$$

All parameters were estimated by maximum likelihood.

For both analyses (day-4 and day-14), four different models were fitted, the first with separate dose response relations for each of the 4 treatment groups (CF_1 vs. CF_2 vs. SF_1 vs. SF_2), the second with combined CF groups (CF_{12}) vs. combined SF groups (SF_{12}), the third with combined exp. 1 groups vs. exp. 2 groups, and the fourth with all groups combined. The corrected Akaike Information Criterium (AIC_c) (Hurvich and Tsai, 1989) was used to decide which model explained the data best and whether different dose response relations should be adopted for (combined) treatment groups.

To assess the effect of MCFA feed supplementation on technical performance, BWG and feed intake during the period of individual housing (>14 days of age) were recorded. In exp. 1, clinical signs, observed in SF broilers around 1 week of age, resulted in 47 gram lower BW compared to CF broilers at 14 days of age (linear regression, with sex correction: $P<0.001$). Because a reduced BW at the start of a recording period is likely to affect the subsequent growth rate (Leeson and Zubair, 1997), data of exp. 1 was considered unsuitable to study the effect on technical performance. In exp. 2, diarrhea also occurred around 1 week of age, with similar frequencies in both treatment groups (14/22 CF groups and 5/10 SF groups;

Fisher exact test: P=0.70), suggesting that the symptoms observed in exp. 1 were not feed related. As BW at 14 days of age were not affected by diarrhea (P=0.75) or feed treatment (P=0.52) in exp. 2 (linear regression, with sex correction: P<0.001), data of this experiment were used to study the effect on technical performance. A broiler that died during the experiment and a broiler that was lame during the last few days of the experiment were excluded from this analysis. In the experiment, 69 broilers, which were detected colonized before or at 4 days PI, were weighed and sexed at 28 days of age, while the remaining 84 broilers (19 colonized and 65 non colonized) were weighed and sexed at 32 days of age. To correct for this, age and final colonization status were included as dependent variables, next to the variables sex and feed treatment, resulting in the equations

$$BWG_i = \beta_0 + \beta_1 Feed_{i1} + \beta_2 Sex_{i2} + \beta_3 Age_{i3} + \beta_4 Col_{i4} + \varepsilon_i \quad (3),$$

and,

$$FCR_j = \beta_0 + \beta_1 Feed_{j1} + \beta_2 Sex_{j2} + \beta_3 Age_{j3} + \beta_4 Col_{j4} + \varepsilon_j \quad (4).$$

Linear regression analyses (SPSS 15.0.1.) were performed to assess if feed treatment affected BWG and FCR significantly.

Prior to inoculation, some groups of broilers got diarrhea at 1 week of age. To study the possible confounding effect of diarrhea on susceptibility, data of exp. 2 were analyzed as described above, in 4 groups, with diarrhea status instead of experiment. AIC_c was used to assess if the occurrence of this symptom was associated with an increased or decreased susceptibility to *Campylobacter* colonization.

RESULTS

Colonization

For each sampling moment, the number of colonized broilers is shown in tables 1 and 2. In exp. 1, 35 of 46 colonized birds, and in exp. 2, 72 of 89 colonized birds were detected *Campylobacter*-positive in the first 4 days PI. In exp. 1, the sentinels remained negative, but in exp. 2, 4 of 16 sentinels were detected *Campylobacter*-positive, at days 7, 8 and 14 days PI. Four birds in exp. 1 and one bird in exp. 2 died after day 4 PI.

Table 1. Sampling result exp. 1.
Results for bacterial culture of fecal samples collected at different time points post inoculation. Rows represent different feed treatment groups (CF = control feed; SF = supplemented feed) and *C. jejuni* C356P inoculation doses (^{10}Log cfu/broiler). Nominators in cells express the number of broilers detected *Campylobacter*-positive, and denominators express the number of broilers sampled.

treatment	Dose	Time point (days post-inoculation)									
group	C356P	1	2	3	4	7	8	9	11	14	Total
CF	-	0/4	0/4	0/4	0/16	0/4	0/16	0/4	0/16	0/16	0/16
CF	1.19	0/4	0/5	0/5	0/33	0/5	0/33	0/5	0/32	1/32	1/33[a]
	2.19	0/5	0/5	2/4	7/31	0/3	2/24	0/2	1/22	1/21	13/33[b]
	3.19	0/5	0/4	4/5	16/28	0/2	0/11	0/2	1/11	0/10	21/32[c]
SF	2.19	0/3	0/3	0/3	1/21	0/3	0/20	0/3	0/20	1/20	2/21
	4.49	0/3	0/3	0/3	5/21	1/3	3/14	0/2	0/11	0/11	9/21[a]
Totals:		0/24	0/24	6/24	29/150	1/20	5/118	0/18	2/112	3/110	

[a] A broiler died after being tested negative at 8 dpi.
[b] A broiler died after being tested positive at 4 dpi.
[c] A broiler died after being tested negative at 7 dpi.

Dose Response: Effect on Susceptibility

The best day-4 model (lowest AIC_c) included separate dose response relations for all four treatment groups (CF_1, CF_2, SF_1, and SF_2). The dose response relations of CF_1, CF_2, and SF_2 show clear sigmoid shapes, whereas the dose response relation of SF_1 is flatter. Generally, confidence intervals are wide outside the range of observations (figure 1). CD_{50} for CF_1 and CF_2 were 2.8 ^{10}Log (95% CI: 2.6-3.3) and 2.1 (95% CI: 1.8-2.3), respectively. CD_{50} for SF_1 and SF_2 were 8.5 ^{10}Log (95% CI: 4.8 - ∞) and 4.7 (95% CI: 4.2-5.2), respectively (figure 1). The AIC_c of alternative models were at least 12.2 higher.

The best day-14 model (lowest AIC_c) included separate dose response relation for CF_{12} and SF_{12}, indicating equal relations in both experiments. Dose response relations of CF_{12} and SF_{12} show parallel sigmoid shapes (figure 2), with the CF_{12} curve positioned approximately in the middle of the two separate CF curves in the final day-4 model (figure 1), and the SF_{12} curve positioned close to the SF curve for exp. 2. CD_{50} for CF_{12} was 2.5 ^{10}Log (95% CI: 2.2-2.8) and CD_{50} for SF_{12} was 4.8 ^{10}Log (95% CI: 4.4-5.2) (figure 2). AIC_c of the alternative models were at least 3.95 higher.

Table 2. Sampling result exp. 2.
Results for bacterial culture of fecal samples collected at different time points post inoculation. Rows represent different feed treatment groups (CF = control feed; SF = supplemented feed) and *C. jejuni* C356P inoculation doses (^{10}Log cfu/broiler). Nominators in cells express the number of broilers detected *Campylobacter*-positive, and denominators express the number of broilers sampled.

treatment	Dose	Time point (days post-inoculation)									
group	C356P	1	2	3	4	7	8	9	11	14	Total
CF	-	0/4	0/4	0/4	0/16	1/4	2/15	0/4	0/13	1/13	4/16
CF	1.47	0/4	3/4	0/4	5/29	0/4	4/24	0/1	0/20	1/20	13/32
	2.47	3/4	2/4	2/4	14/26	0/2	1/12	0/0	1/11	2/10	25/33
	3.47	2/4	4/4	4/4	20/22	0/0	1/2	0/0	0/1	0/1	31/32
SF	2.47	0/1	0/2	0/1	0/10	0/1	1/10	0/2	1/9	0/8	2/10
	3.47	0/2	0/1	0/2	2/11	0/1	0/9	0/1	0/9	0/9	2/11
	4.47	0/1	0/2	0/1	2/10	0/0	0/7	0/2	0/7	1/7	3/10[a]
	5.47	1/2	0/1	1/2	7/9	0/0	0/2	0/0	0/2	0/2	9/11
Totals:		6/22	9/22	7/22	50/133	1/12	9/81	0/9	2/72	5/70	

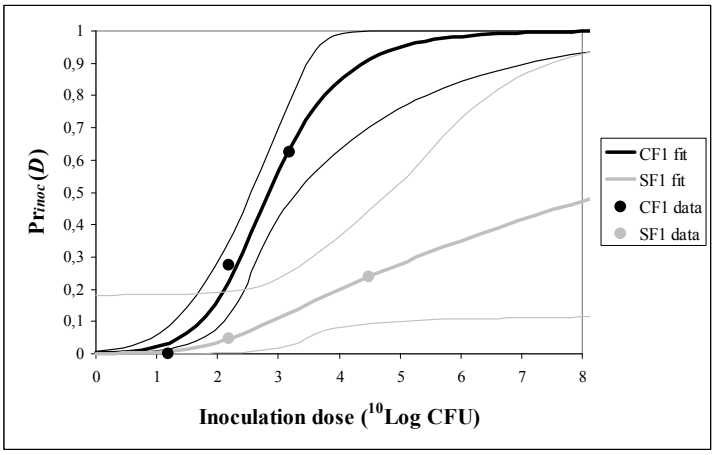
[a] A broiler died after being tested negative at 4 dpi.

In separate analyses of data of exp. 2 inclusion of a variable describing whether a broiler originated from a group with diarrhea did not result in a better fit, with AIC_c being approx. 4 points higher in both the day-4 and day-14 models.

Effect on Technical Performance

BWG was estimated 49±24 g higher in broilers with were provided supplemented feed (P=0.044) when correcting for the effect of sex (P<0.001), age (P<0.001), and final colonization status (P=0.398). FCR was estimated 0.061±0.034 lower in broilers with were provided supplemented feed (P=0.075) when correcting for sex (P=0.179), age (P=0.183), and final colonization status (P=0.735).

a



b

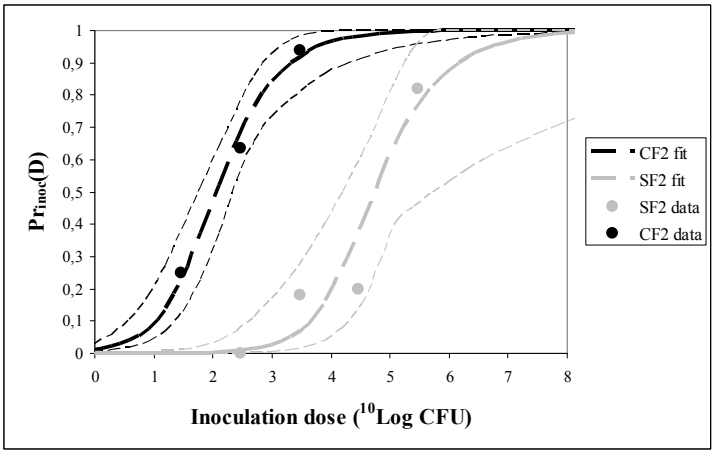


Figure 1. Dose response curves for the final model of the day-4 analysis
The relation between inoculation dose en the probability of colonization occurring as a result of inoculation, $Pr_{inoc}(D)$, up to 4 days PI, for exp. 1 (a) and exp. 2 (b). Best fitted curves, with 95% confidence bounds, represent relations for control feed (CF_1 or CF_2) and MCFA supplemented feed (SF_1 or SF_2) fed broilers. Dots represent raw data as observed in the experiments.

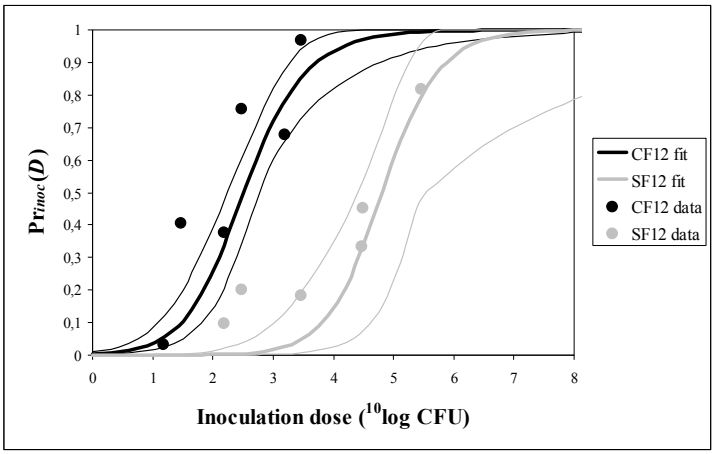


Figure 2. Dose response curves for the final model of the day-14 analysis
The relation between inoculation dose en the probability of colonization occurring as a result of inoculation, $Pr_{inoc}(D)$, up to 14 days PI, for combined experiments (1 and 2). Best fitted curves, with 95% confidence bounds, represent relations for control feed (CF_{12}) and MCFA supplemented feed (SF_{12}) fed broilers. Dots represent raw data as observed in the experiments.

DISCUSSION AND CONCLUSION

The aim of this study was to determine the effect of MCFA feed supplementation on the susceptibility of broilers for colonization with *C. jejuni*, and on technical performance. The effect on susceptibility was assessed by determining the relation between the *Campylobacter* inoculation dose and the subsequent occurrence of colonization. Dose response curves of SF treatment groups were shifted to the right, indicating that SF broilers required a higher inoculation dose to become colonized than CF broilers (figures 1 and 2). Analysis of technical performance showed that BWG was increased in SF broilers, while FCR was not affected.

In the final day-4 model, the dose response curve of SF_1 was almost flat with wide confidence bounds (figure 1), in contrast to the other dose response curves (CF_1, CF_2, and SF_2). The reason for this is likely the lack of data on doses that would have resulted in high percentages of colonized broilers. This is corroborated by the final model of the day-14 analysis, where the SF_{12} curve was similarly shaped as and closely positioned to the SF_2 dose response curve from the day-4 model, with similar confidence bounds. To obtain accurate estimates of dose response relations

it is clearly important to choose inoculation doses that result in both low and high probabilities of colonization.

As the period between inoculation and detectable shedding of *Campylobacter* might exceed 4 days in some individuals, data of the day-14 analysis is more likely to include all broilers colonized due to inoculation. Considering the day-14 analysis corrected for the occurrence of colonization by transmission, this analysis could be considered superior. The day-4 analysis did however result in similarly shaped and positioned dose response curves, and a similar conclusion on the effect of MCFA feed supplementation. Because this similarity suggests that most broilers colonized after 4 days PI were colonized by transmission, we state that, based on the results in this study, challenge experiments not necessarily have to last longer than 4 days.

In exp. 1, clinical signs (diarrhea, depression and growth retardation) that occurred in SF broilers around 1 week of age was absent in CF broilers. This might have affected susceptibility. To assess if bias was likely, in exp. 2, broilers were housed in multiple groups and the occurrence of clinical signs during the first two weeks was recorded for each group. During this period diarrhea was observed in CF and SF broilers with similar frequency. Because separate analyses on the effect of diarrhea on susceptibility did not result in better fitting models, we conclude that the occurrence of diarrhea most likely was not affecting susceptibility.

The observed positive effect on BWG might have been caused by antimicrobial effects of the fatty acids, as feed supplementation with Capric Acid and Lauric acid, two MCFAs, has been shown to decrease the concentration of *Clostridium perfringens* in jejunum and ileum of *C. perfringens* challenged broilers (Jansman et al., 2006). Although feed supplementation with antimicrobial agents has the potential to improve feed efficiency (Dibner and Richards, 2005; Jansman et al., 2006), in this study no significant effect on FCR was observed.

Although the mechanism by which MCFA supplementation reduces susceptibility could not be revealed within the experimental design in this study, in the final day-14 model the *C. jejuni* dose necessary to colonize 50% of inoculated broilers was 200 times higher in broilers fed with supplemented feed than in control broilers. This effect on susceptibility and the positive effect on BWG are promising findings for the implementation of MCFA feed supplementation as an intervention for reduction of susceptibility in broilers. Because the *Campylobacter* exposure dose that broilers experience in the field is unknown, field trials are necessary to

determine to what extent MCFA supplementation reduces *Campylobacter* colonization in the field.

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Chapter 5

Effect of volatile fatty acid on *Campylobacter jejuni* levels in crop and ceca of broilers

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Submitted

ABSTRACT

During the slaughter process, poultry meat may become contaminated with *Campylobacter* due to cross contamination with bacteria from the digestive tract of colonized broilers. The bacteria may be localized anywhere between the crop and the ceca. Reduction of the amount of *Campylobacter* in the lumen of these organs might contribute to reduced carcass contamination, thereby limiting human exposure. The aims of this study were to quantify the effect of drinking water supplementation with volatile fatty acid on *Campylobacter* colonization levels in crop and ceca, and to assess the correlation in numbers of bacteria between samples from these two sites to determine whether results from one sample are predictive for the level of colonization in the other.

Two replicate experiments were carried out. In each experiment 24 individually housed *Campylobacter*-colonized broilers were provided with water containing 0.2% volatile fatty acids or non-supplemented water. Three days later, the birds were killed and the content of crop and ceca was cultured on modified charcoal cefoperazone desoxycholate agar plates to determine *Campylobacter* levels.

Volatile fatty acid supplementation did not result in a significant reduction of colonization levels neither in the crop ($p=0.50$) nor in the ceca ($p=0.92$). Pearson's correlation coefficient (ρ) did not reveal a correlation between crop and cecal colonization levels ($\rho=0.071$; $p=0.69$). These findings indicate that this treatment, applied shortly before slaughtering, does not contribute to reduction of *Campylobacter* levels, and is unlikely to reduce carcass contamination. Moreover, the latter finding emphasizes the desirability to include the assessment of crop colonization levels in future studies on cross-contamination during slaughtering.

INTRODUCTION

Campylobacter spp are a common cause of diarrhea in humans, and human infection is associated with the handling and consumption of contaminated poultry meat (Friedman et al., 2004). Risk assessment studies have indicated that reduced *Campylobacter* colonization levels at slaughtering could result in reduced contamination levels of poultry carcasses and meat, which in turn reduces the risk of infection in humans (Nauta et al., 2009a; Rosenquist et al., 2003).

Oral application of medium chain fatty acids (de Los Santos et al., 2008), bacteriocins (Stern et al., 2005), and bacteriophages (Wagenaar et al., 2005) have shown to reduce cecal colonization levels in broilers. Cecal colonization levels do, however, poorly predict the level of carcass contamination (Allen et al., 2007; Nauta et al., 2009b; Stern et al., 2003). One explanation is that cross-contamination originates from other parts of the digestive tract, e.g. the crop, which has been shown to contain 4.8-5.0 ^{10}Log cfu *Campylobacter* at slaughtering (Smith et al., 2006). The crop is more likely to rupture during evisceration than intestines (Hargis et al., 1995), and the content may easily spread out over the carcass, as it is rather fluid (Byrd et al., 2002).

It might, however, be difficult to determine whether contamination originates from ceca or crop. Therefore, the effect of an intervention measure aimed at reducing the amount of *Campylobacter* in the digestive tract should preferably be determined not only by quantifying bacterial levels in cecal content but also in crop content.

One of the potential intervention measures that can be applied shortly before slaughtering is oral application of volatile fatty acids (VFA, C<6), e.g. formic acid and acetic acid, which are active against many bacteria, including *Salmonella* spp. (McHan et al., 1993; Thompson et al., 1997) and *Campylobacter* spp. (Chaveerach et al., 2002). A negative side effect of drinking water supplementation with 0.5% formic or acetic acid was, however, reduced water consumption in broilers (Byrd et al., 2001), which might have resulted in reduced bodyweight at slaughtering. Lower doses may improve this side effect, and may still be effective in reducing *Campylobacter* levels.

The current study was carried out to examine the effect of drinking water supplementation with 0.2% VFA on crop and cecal colonization levels. In addition, the correlation between crop and cecal colonization levels was determined to assess

whether colonization levels in a sample from one organ was predictive for colonization levels of the sample from the other organ.

MATERIALS AND METHODS

Experimental design

Two replicate experiments were carried out subsequently. *Campylobacter*-colonized broilers originated from previous experiments in which commercial broilers (Ross 308) were inoculated with *Campylobacter jejuni* C356P at 14 (experiment 1) or 18 days (experiment 2) of age (van Gerwe et al., submitted). Birds were housed individually from 14 days of age to provide independence of the observations. Birds were housed on litter in 40x40 cm cages, and had access to water and a mash diet free from antibiotics and anti-coccidia drug (main components: wheat (36.5%), soy (21.2%), corn (20%), peas (15%), and soy-oil (3.95%)).

At 28 days of age, birds were weighed and randomly allocated to two treatment groups, each consisting of 12 broilers. This experimental set up provided 90% statistical power to detect 1.5 ^{10}Log colony forming units (cfu) change in colonization levels, with expected standard deviation (SD) of 1.25 ^{10}Log cfu (Musgrove et al., 2001; Smith et al., 2006), and alpha error 5% (Win Episcope 2, www.clive.ed.ac.uk/winepiscope). Experiments were approved by the animal ethical committee of Utrecht University.

Treatment

In each experiment, one group was provided with drinking water supplemented with 0.2% (g/g) of a blend, which consisted of ammoniated formic acid (90%) and acetic acid (10%) The other group (controls) received non supplemented drinking water. Water was provided in drinking cups, and refreshed twice daily.

Sampling and testing

At 31 days of age, feed was withdrawn, while access to water continued. After 6 to 8 hours, birds were euthanized by injection of T 61® (Intervet B.V., the Netherlands), weighed and sexed. Ceca were removed from the carcass. Ligatures were placed on the oesophagus proximal and distal of the crop, and the crop was dissected. Subsequently, crop surface was sprayed with 70% ethanol and 2 ml saline was injected into the crop, which typically contained less than 1 ml fluid

content. Crops and ceca were transported to the laboratory and processed within an hour.

Enumeration of cecal and crop content, expressed in ^{10}Log cfu/gram, was performed by plating 10-fold dilutions (PBS) on modified charcoal cefoperazone deoxycholate agar plates (Biotrading Benelux B.V., Mijdrecht, the Netherlands). All plates were incubated micro-aerobically at 37°C, and examined for the presence of *Campylobacter*-suspect colonies after 48 hours. Microscopic examination of morphology and motility was used as confirmation.

Statistical analyses

Culturing of crop content was expected to result in at least some samples below the detection limit, further referred to as 'negative' results. A cohort analysis stratified on experiment was conducted to determine if the relative risk for crops of treated bird to be *Campylobacter*-negative was affected by experiment. Depending on the result (Breslow-Day statistic), colonization levels in crops of treated birds and controls were compared for separate or pooled experiments, using Mann Whitney test. Cecal colonization levels in treated birds and control were compared by linear regression, with treatment and experiment as predictive variables, according to the equation:

$$Qcecal_i = \beta_0 + \beta_1 exp_{i1} + \beta_2 treatment_{i2} + \varepsilon_i.$$

Broilers in experiment 2 were heavier at the start of the treatment, which is likely to affect BWG. Sex also affects BWG. Therefore, the effect of VFA on BWG was assessed by linear regression, with experiment, sex and treatment as predictive variables, according to the equation:

$$BWG_i = \beta_0 + \beta_1 exp_{i1} + \beta_2 Sex_{i2} + \beta_3 treatment_{i3} + \varepsilon_i.$$

The correlation between colonization levels was assessed by calculating Pearson's correlation coefficient (ρ) and testing whether ρ was significantly larger than zero. ρ was determined for treated broilers, controls, and pooled data. ρ could only be calculated for broilers with positive test results (expressed as ^{10}Log cfu/g) for both sample types, as quantitative estimates are required. The exclusion of broilers with negative test might have biased the estimation. To assess if such bias was likely to occur, cecal colonization levels were compared for broilers with positive and negative crop results, using Student's *t*-test.

RESULTS

In total, cecal samples from 47 broilers were included in the analyses, as one broiler died before the end of the trial. Crop samples were obtained from 44 birds, as three crops ruptured at sampling or could not be analyzed due to overgrowth with concurrent bacterial flora. Cecal contents of all birds were *Campylobacter*-positive. In the treatment group, *Campylobacter* was detected in 6 of 10 (exp. 1) and 10 of 11 (exp. 2) crops. In controls, *Campylobacter* was detected in 8 of 12 (exp. 1) and 12 of 12 (exp. 2) crops. These result did not differ significantly between the two experiments (Breslow-Day statistic: p=0.389). Pooling of the results from the two trials was therefore considered legitimate. No difference in crop colonization levels between control and treated broilers was observed (p=0.50) (Table 1).

Experiment did not have a significant effect on cecal colonization levels (p=0.11), and the cecal colonization levels did not differ significantly between control and treated broilers (p=0.92) (Table 1). Treatment did not have a significant effect on BWG, corrected for experiment (p<0.001) and sex (p=0.023) (p=0.87).

Table 1. Effect of 0.2% volatile fatty acid drinking water treatment during 3 days on colonization levels in crop and ceca of 31-day-old broilers (combined data of 2 replicate experiments).

Treatment	No. crops	Median Campylobacter count and range (^{10}Log cfu /gram)	No. ceca	Mean Campylobacter count ± SD (^{10}Log cfu /gram)
no	23	4.8 (n.d.[1]-6.5)	24	8.5 ± 0.7
yes	21	4.0 (n.d.[1]-6.6)	23	8.5 ± 0.7
test result:		p = 0.50 (Mann Whitney)		p = 0.92 (Linear regression)

[1] n.d.= *Campylobacter* not detected, with a detection limit of app. 0.8 ^{10}Log /gram crop content.

Cecal and crop colonization levels were not correlated neither for treated broilers (n=16; ρ=0.06, p=0.84) nor for controls (n=19; 0.09, p=0.71). Similarly, pooled data did not show a correlation between samples from both organs (ρ=0.07, p=0.69). No difference between cecal colonization levels in broilers with negative crop results (n=9) and broilers with positive crop results (n=36) was observed (p=0.70). This finding indicates that if the crop colonization levels of the 9 'negative' broilers would have been known, correlation most probably would have remained lacking.

DISCUSSION AND CONCLUSION

The aims of this study were to quantify the effect of drinking water supplementation with VFA on crop and cecal *Campylobacter* colonization levels, and to determine the correlation between crop and cecal colonization levels. Water supplementation with 0.2% VFA during three days did not result in a significant reduction of colonization levels in both crops and ceca of *Campylobacter*-colonized broilers. This implies that this treatment will most likely not contribute to a reduction of carcass contamination during slaughtering.

In this study, we used individually housed broilers to prevent transmission of *Campylobacter*. Consequently, each individual bird could be considered an independent observational unit, thereby justifying the use of the statistical methods applied. The two experiments provided comparable results, thereby allowing for pooling of the data which increases the power of the study. Studies in which broilers were housed in groups showed considerable variation between replicate experiments (de Los Santos et al., 2008; de los Santos et al., 2009), indicating that the experimental design in our study is more appropriate as the broiler is the unit of observation. Of course, we do realize that group housing is more representative for the field. Evaluation using individually housed broilers, however, requires fewer animals which is preferred when determining proof of principle.

We only tested content of ceca and crop, and did not investigate from which part of the digestive tract cross-contamination of the carcass originates. Efficacy of interventions that reduce *Campylobacter* colonization levels depends on the correlation between colonization levels of intestines or crops and carcass contamination levels. Most studies did not find a correlation between cecal colonization and carcass contamination levels (Allen et al., 2007; Nauta et al., 2009b; Stern et al., 2003), possibly because the contamination originated from crop content. A correlation between crop colonization levels and carcass contamination levels has not been studied thus far. Although the total amount of *Campylobacter* was considerably lower in crops than in ceca (Table 1). Crop colonization levels observed in this study were similar to levels quantified by (Smith et al., 2006), which are sufficiently high to cause carcass contamination levels previously observed (median 4.8 ^{10}Log cfu/carcass) (Stern et al., 2003). The fluid character of crop content and the higher likelihood of rupturing (Hargis et al., 1995) may facilitate the spread of crop content over the carcass. Although the role of the crop as source for carcass contamination has not been demonstrated yet, crop content should be considered a potentially contributing source for cross-contamination. The

data collected in this study did not demonstrate a correlation between cecal and crop colonization levels, which implies that when studying the sources of contamination in the slaughterhouse, content from both organs should be examined.

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Chapter 6

Experimental *Campylobacter* colonization in chicken embryo intestines

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ABSTRACT

Colonization properties of *C. jejuni* strains are usually evaluated in chickens. As an alternative for this animal model, a colonization model in chicken embryos has been suggested. Such a model might resemble, to a certain level, the micro-environmental conditions in young chickens, and might allow for the evaluation of colonization potential and bacterium-host interaction in absence of gut flora. Moreover, studies on detection of non-culturable *Campylobacter* cells in embryonated chicken eggs show that such model has a high analytical sensitivity.

To assess the value of this embryo colonization model, embryonated eggs were injected into the amniotic fluid with 0.85, 1.85, or 2.85 ^{10}Log cfu *C. jejuni* at 16 days of incubation. Colonization levels of *Campylobacter* in intestines were determined at day 1, 2 and 3 post inoculation (PI). Duodenum and cecum showed a positive correlation between inoculation dose and colonization level at 1 day PI. Maximal colonization levels were reached 2 days PI, and remained constant till 3 days PI, with median values 3.4 ^{10}Log cfu/g in duodenum and 6.7 ^{10}Log cfu/g in cecum. Median level in cloacal content, assessed at 3 days PI, was 8.0 ^{10}Log cfu/ml.

Analytical sensitivity, defined as the probability of colonization following inoculation of one cfu *C. jejuni*, was assessed for two *C. jejuni* strains with different *in vivo* colonization potential. Embryonated eggs were inoculated with very low doses of a lab-adjusted and a bird-passaged *C. jejuni* strain, and intestinal colonization was determined qualitatively at 3 days PI by culture of cloacal swabs. Analytical sensitivity was estimated to be 0.35-0.7, for both strains.

In conclusion, the model is a sensitive tool for the detection of *Campylobacter* which provides conditions for a steady growth with colonization levels in cloacal content comparable to colonization in live birds.

INTRODUCTION

Campylobacter jejuni is an important cause of gastrointestinal disease in human. Human infections are often caused by contaminated poultry meat (Friedman et al., 2004). Therefore, intervention strategies are aiming for prevention of colonization in broilers (Wagenaar et al., 2006). The development of targeted strategies to control *Campylobacter* in broilers, like vaccine development, requires knowledge of those bacterial factors essential for colonization of the avian gut and the interaction with intestinal epithelial cells. Therefore, variation in *Campylobacter* colonization potential has been studied in chicken (Cappelier et al., 1999a; Hanel et al., 2008; Korolik et al., 1998; Muller et al., 2006; Ringoir et al., 2003), and host-pathogen interaction has been studied in intestinal chicken cells (Borrmann et al., 2007; Byrne et al., 2007; Li et al., 2008; Van Alphen et al., 2008). Although *Campylobacter* studies in live animals and in cell cultures are useful, both systems have some disadvantages. Studying the colonization process in live animals is complex due to the influence of gut flora, while chicken cell cultures represent a simplification of the intestinal tract that does not allow for studying complex host-pathogen interaction like chemotaxis or motility. A model with both the complex structure of the avian gut and the germ-free environment of chicken cell cultures offers a tool for research on aspects of colonization potential and host-pathogen interaction. The chicken embryo at the last stage of incubation provides these circumstances: the gut content contains no micro-organisms and the gut is close to the level of maturation of a hatchling.

In previous studies, embryonated chicken eggs have been used to study the viability of *C. jejuni* strains (Cappelier et al., 1999b; Chaveerach et al., 2003). In these studies, the yolk sac or the amniotic sac was inoculated at 7 or 9 days of incubation, and *Campylobacter* was recovered from amniotic or yolk fluid 0.5 to 4 days later. Although colonization of embryonic intestines was not assessed in these studies, the observed recovery of nonculturable *C. jejuni* strains suggests that recovery in embryos might be a sensitive diagnostic tool for the detection of viable *Campylobacter* cells. When embryonated chicken eggs are inoculated in the amniotic sac in the last few days before hatching, colonization of the embryonic intestines might occur as the amniotic fluid is directly connected to the intestinal tract (Jochemsen et al., 2002).

Because embryos in the last few days of incubation might be a good colonization model and a sensitive detection tool, we assessed *Campylobacter* colonization characteristics with the aim to explore (i) how colonization levels develop over

time, (ii) if a quantitative dose response relation could be observed, and (iii) to assess the analytical sensitivity of the model for strains with different *in vivo* colonization ability.

MATERIAL AND METHODS

In this study four experiments were carried out. In experiments 1 and 2, colonization levels were determined at 1, 2, and 3 days PI in different parts of the embryonic intestines to assess when maximal colonization levels were reached and how long they were maintained. It was also determined whether the inoculation dose affected observed colonization levels. In experiment 3 and 4, the relation between inoculation dose and the probability of colonization was assessed, to estimate the analytical sensitivity of the model, which was defined as the probability of colonization following inoculation of one cfu *C. jejuni*. In experiment 4, two strains with different *in vivo* colonization potential were used to explore if the embryo model's analytical sensitivity was affected by *in vivo* colonization potential.

Incubation of eggs

Hatching eggs originating from Specified Pathogen Free broiler breeder flocks (Intervet Schering-Plough Animal Health, Boxmeer, the Netherlands) were used. The *Campylobacter* status of the breeder flocks was determined shortly after hatching eggs were laid, based on 25 cloacal swabs per flock, which was sufficient for detection of a minimum prevalence of 10% with a probability of 95% (Dohoo et al., 2003). All flocks tested negative for *Campylobacter*. Eggs weighing 48 to 69 grams were incubated (Pas Reform type C41), with egg shell temperatures around 37.8°C throughout the experiments. Relative humidity was 65% in experiments 1 and 2, and was changed to 80% in experiments 3 and 4 to increase amniotic sac volume, which would most likely facilitate inoculation into this compartment. Eggs were turned automatically every hour. After 9 days of incubation, eggs were candled and only vascularised eggs were used for further processing.

Inoculation, sampling and microbiology

At day 16 of incubation, eggs were sprayed with 70% ethanol. A small hole was made in the blunt side of the egg using an eggshell punch. Inoculation was performed with needles of different lengths, to optimize injection into the amniotic fluid: eggs with initial weight of 48-62 grams were inoculated using 1¼ inch needles (Neolus 0.7 x 30 mm, NN-2232R, Terumo) and eggs weighing 63-69 g

were inoculated using 1½ inch needles (Neolus 0.9 x 40 mm, NN-2038R, Terumo). *Campylobacter* cultures in Heart Infusion Broth (37°C, micro-aerobically, shaken at 160 gyrations/min for 15 hours) were diluted with PBS, to obtain intended inoculation doses, and kept at room temperature. Eggs were randomly assigned to dose groups and 0.05 ml of the *Campylobacter*-suspension was inoculated in eggs within half an hour after preparation of the inoculums. Needles were inserted up to the conus, directing towards the centre of the egg. The concentration of *Campylobacter* in the administered inoculums was determined by plating on *Campylobacter* agar plates (*Campylobacter* agar base Oxoid CM 271, 4% saponin-lysed horse blood; Biotrading Benelux B.V., Mijdrecht, the Netherlands). Directly after inoculation, incubation of eggs was continued. Negative control eggs were not inoculated. Number of eggs, inoculation doses, and sampling procedures are described in the concerning sections below. Samples were cultured on *Campylobacter* agar plates at 37°C under micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) and plates were read out after 48 hours. *Campylobacter* growth was confirmed by microscopic examination of suspect colonies.

Colonization levels in embryonic intestinal tract

In experiments 1 and 2, embryos were inoculated with 0.85, 1.85 or 2.85 ^{10}Log cfu *C. jejuni* C356 (Jacobs-Reitsma et al., 1995). At 1, 2, and 3 days PI, 5 embryos from each dose group and a negative control embryo were sampled. The egg shell was removed, the embryo was euthanized by cervical dislocation, and sprayed with 70% ethanol. After opening the body cavity, the duodenal loop was removed, liquid cloaca content was sampled using a syringe with a needle (sufficient volume at day 3 PI only), and the ceca were removed. Duodenum and ceca were homogenized (Turrax 18, Janke und Kankel, Breisgau, Germany), and eight tenfold dilutions of homogenized intestines and cloacal fluid were made in PBS.

Samples were plated on *Campylobacter* Agar plates using a track-dilution method (Jett et al., 1997), with theoretical detection limits of 2 ^{10}Log cfu/g for intestinal samples and 2 ^{10}Log cfu/ ml for cloacal fluid samples. Negative control eggs were analyzed qualitatively.

Embryos with at least one *Campylobacter* positive sample were considered colonized, and were included in statistical analyses. The results of the three sample types collected at day 3 PI (duodenum, cecum, and cloacal content) were compared using Friedman's test, followed up by Wilcoxon's signed rank test for each combination of two sample types if a significant result would occur.

To determine when and in which sample type a quantitative relation between inoculation dose and colonization level existed, linear regression was applied. A variable *Exp* was added to correct for a possible experimental effect, resulting in the equation:

$$Q_{i\ ,sample\ type,\ t} = \beta_0 + \beta_1 Exp + \beta_2 Dose_{i2} + \varepsilon_i \quad (1)$$

with $Q_{i\ ,sample\ type,\ t}$ as the *Campylobacter* colonization level for a specific sample type and sampling moment, and *Dose* representing the inoculation dose in ^{10}Log cfu. Samples with colonization levels below the detection limit were assumed to equal 1 ^{10}Log cfu/g. Sensitivity analysis on the effect of other possible values (i.e. 0 and 2 ^{10}Log cfu/g) for these samples was performed. Statistical analyses were carried out using SPSS 15.0.

Analytical sensitivity

To study analytical sensitivity of the model, which was defined as the probability of colonization, Pr_+, following inoculation of one cfu *C. jejuni*, two strains were used: *C. jejuni* C356 (Jacobs-Reitsma et al., 1995), which was incapable of colonizing any of 140 individually housed 2-week old commercial broilers inoculated with 1 to 4 ^{10}Log cfu (data not shown), and a bird-passaged C356 strain (C356P), which colonized 50 of 137 individually housed 2-week old commercial broilers inoculated with the same doses (Van Gerwe et al., submitted).

In experiment 3, 3 x 30 embryonated eggs were inoculated with -0.67, -0.17, or 0.33 ^{10}Log cfu C356P, and 9 embryonated eggs served as negative control. In experiment 4, 3 x 30 embryonated eggs were inoculated with -0.33, 0.17, or 0.67 ^{10}Log cfu C356P, 3 x 30 embryonated eggs were inoculated with -0.44, 0.06, or 0.56 ^{10}Log cfu C356, and 12 embryonated eggs served as negative control.

Three days PI, embryos were euthanized by cervical dislocation, and sprayed with 70% ethanol. After drying, swab samples (Copan, Italy), taken from cloacal content of randomly ordered embryos (including negative controls), were directly streaked on *Campylobacter* Agar plates.

The relation between inoculation dose and the occurrence of colonization was assessed by fitting a hypergeometric dose response model (Teunis et al., 2000) on combined data of experiments 3 and 4. According to this model, inoculation with dose *D* results in a probability of colonization which is described as:

$$Pr_{inoc}(D) = 1 - {}_1F_1(\alpha, \alpha+\beta, -D), \quad (2)$$

in which ${}_1F_1$ is the Kummer confluent hypergeometric function, D is the expected number of inoculated pathogens, and α and β are parameters for the Beta-distribution of the probability of colonization following exposure to a single bacterium (Teunis et al., 2000). As most likely not all embryonated eggs were injected into the amniotic sac, a probability of incorrect inoculation, $Pr_{inc\text{-}inoc}$, was included. The probability of colonization of an embryo is defined as:

$$Pr_{+}(D) = (1 - Pr_{inc\text{-}inoc}) * Pr_{inoc}(D), \quad (3)$$

All parameters were estimated by maximum likelihood. Different models were fitted, with $Pr_{inc\text{-}inoc}$ as a constant value or dependent on needle length and/or experiment, and Pr_{inoc} depending on strain and/or experiment. We also fitted models with Pr_{inoc} described as a single-hit model with fixed probability of infection r (Haas, 1983): $Pr_{inoc}(D;r) = 1 - e^{-r \cdot D}$. The corrected Akaike Information Criterium (AIC_c) (Hurvich et al., 1989) was used to decide which model explained the data best.

RESULTS

Colonization levels

All 6 negative control embryos in experiments 1 and 2 tested negative. Sixty-two of 91 inoculated embryos had at least one *Campylobacter* positive sample. From 2 days PI onwards colonization levels of intestinal samples were constant (figure 1), with median values in duodenum and cecum of 3.4 and 6.7 ^{10}Log cfu/g, respectively. At day 3 PI, median colonization levels differed significantly between each of the three sample types ($P<0.05$), with lowest levels in duodenum and highest levels (8.0 ^{10}Log cfu/ml) in cloacal content (figure 1).

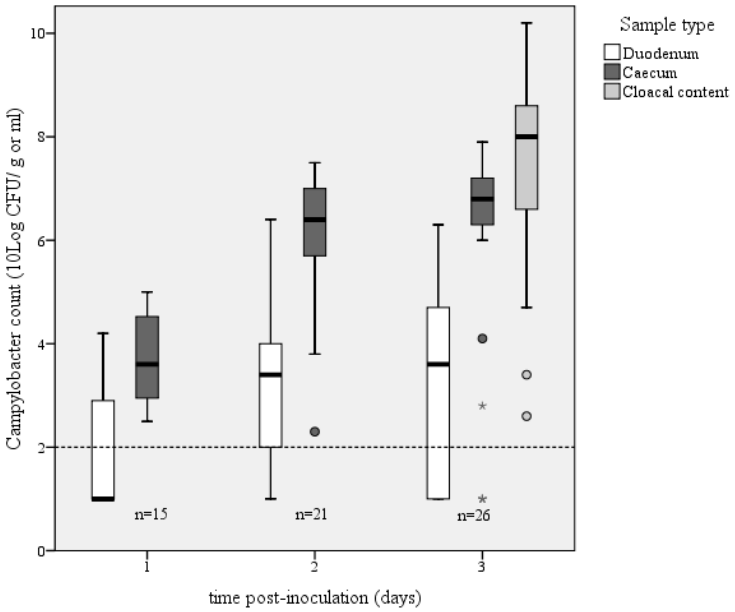

Figure 1. Boxplot of distribution of *Campylobacter* colonization levels (y-axis) in colonized embryos (n=62) at different sampling moments (x-axis). Median, interquartile range, range and outliers (° and *) are shown for combined results from two replicate experiments. Dashed horizontal line represents the detection limit.

Quantitative dose response relation

At day 1 PI, a significant relation between inoculation dose and colonization levels existed for duodenum and cecum samples ($P<0.05$), when corrected for the effect of experiment ($P<0.05$) (figure 2). In duodenum samples, each ^{10}Log cfu increase in inoculation dose resulted in 1.1 (S.E. 0.3) ^{10}Log cfu/g higher colonization levels, while levels differed 1.0 (S.E. 0.3) ^{10}Log cfu/g between replicate experiments. In cecum samples, each ^{10}Log cfu increase in inoculation dose resulted in 0.9 (S.E. 0.2) ^{10}Log cfu/g higher colonization levels, while levels differed 1.3 (S.E. 0.3) ^{10}Log cfu/g between replicate experiments. Other values for samples under the detection limit (0 and 2 ^{10}Log cfu/g) did not change the outcome of the analysis. At days 2 and 3 PI, inoculation doses and colonization levels were not significantly related ($P>0.10$).

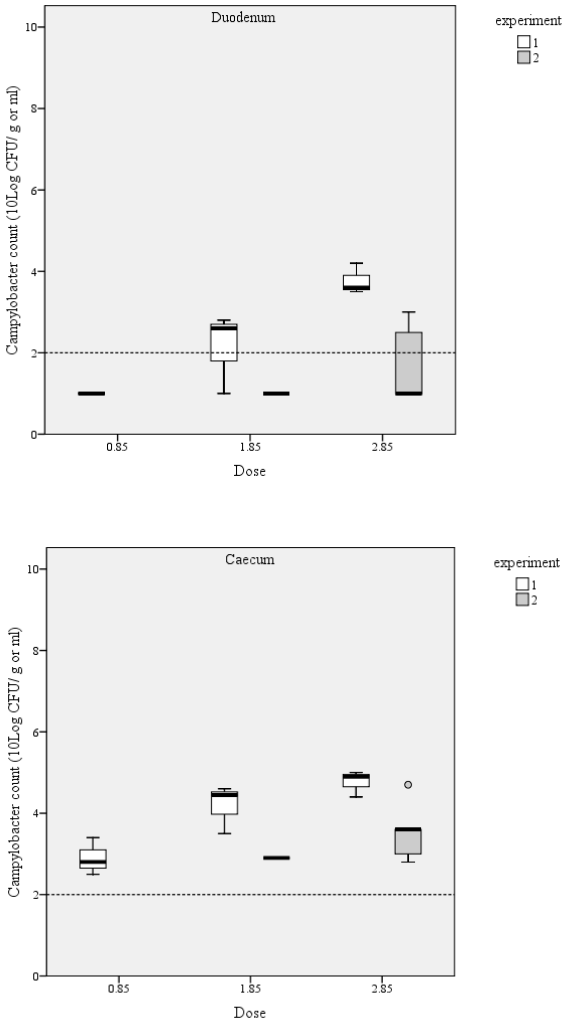

Figure 2. Boxplots of distribution of *Campylobacter* colonization levels (y-axis) in duodenal wall samples (a) and cecal wall samples (b) of colonized embryos 1 day PI (n=15). Each boxplot represents a group of embryos exposed to 0.85, 1.85, or 2.85 ^{10}Log cfu/embryo (x-axis), in each of two replicate experiments. Median, interquartile range, range and outliers (°) are shown. Dashed horizontal lines represent the detection limit.

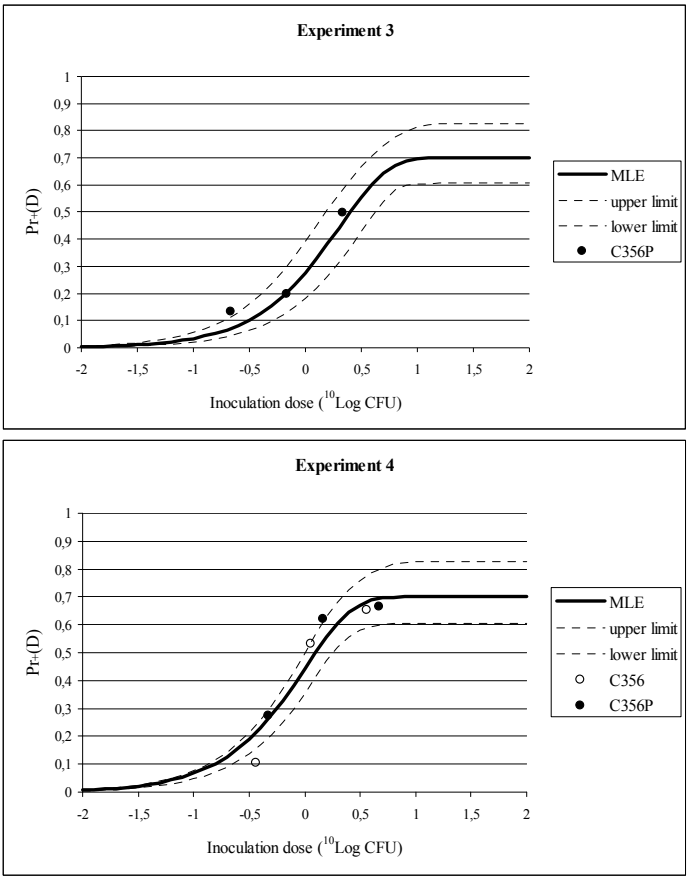

Figure 3. Observed data and Maximum Likelihood Estimate (MLE) and 95% confidence limits of fitted dose response curve for the probability of an embryo being detected colonized, $Pr_{+}(D)$, following inoculation with (a) C356P (experiment 3) or (b) C356 and C356P (experiment 4).

Analytical Sensitivity

All 21 negative control embryos in experiments 3 and 4 tested negative. Due to the death of 4 embryos in exp. 4, 176 of 180 inoculated embryos were included in the analysis. Proportions of colonized embryos per dose group are shown in figure 3. In the final model for $Pr_{+}(D)$, the probability of incorrect inoculation ($P_{inc\text{-}ino}$) was estimated to be 0.30 (95% CI: 0.17-0.39), independent of needle length or experiment. The probability of colonization following inoculation, $Pr_{inoc}(D)$, was defined as $Pr_{inoc} = 1-(1-r)^{n}$ (Teunis et al., 2000), with dose n, and constant r, which

did not vary between the two strains (C356 and C356P) and was estimated 0.50 (95% CI: 0.29-0.86) for experiment 3, and 1 (95%: 0.65-1) for experiment 4, resulting in dose response curves for separate experiments (figure 3a & b). AICs of other models were at least 1.06 higher than the final model, indicating a worse fit. For inoculation of one cfu, Pr_{inoc} is equal to r. Therefore, the analytical sensitivity, which is equal to $(1- P_{inc-ino})*r$, was estimated 0.35 for experiment 3 and 0.7 for experiment 4.

DISCUSSION AND CONCLUSION

Injection of *Campylobacter* into the amniotic sac is an indirect method for inoculation of the gastrointestinal tract of the chicken embryo, which is characterized by the absence of gut flora, and weakly acid gizzard content (pH ≈ 5; data now shown). After inoculation of C356, colonization levels in duodenum and ceca did not change between day 2 and 3 PI, suggesting the availability of sufficient nutrients to ensure a steady growth. Highest colonization levels, detected in cloacal fluid (median: 8.0 ^{10}Log cfu/ml), were similar to cecal levels commonly observed *in vivo* (Knudsen et al., 2006; Shanker et al., 1988).

A positive correlation between inoculation dose and colonization levels was observed at day 1 PI in duodenum and cecum. This quantitative dose response, which has also been observed in day-old chicks inoculated with *C. jejuni* 81116 WT (Wassenaar et al., 1993), suggests that the chicken embryo model can be used to assess the number of viable *Campylobacter* cells in inoculums.

The analytical sensitivity of the model, expressed as the probability of colonization of one inoculated *Campylobacter* cell was estimated 0.35 in exp. 3 and 0.7 in exp. 4. Both values are orders of magnitude higher than the average value for C356P determined in experiments with 14-18 day-old broilers, being 0.0037 (Van Gerwe et al., submitted). Considering the recovery of non-culturable cells from amniotic fluid in previous studies (Cappelier et al., 1999b; Chaveerach et al., 2003), the model might have diagnostic value for the detection of both fully viable and sublethally damaged, non-culturable, *Campylobacter* cells.

The high analytical sensitivity of the embryo model indicates that inactivation of *C. jejuni* is limited, which might be related to the higher gastric pH in embryos compared to live chickens (pH approx. 3) (Durant et al., 1999; Farner, 1942). The high analytical sensitivity could also be a result of *Campylobacter* growth, or 'enrichment', in the amniotic fluid, followed by translocation of these *Campylobacter* cells to the embryonic gastro-intestinal tract.

Qualitative dose response curves of the poorly colonizing *C. jejuni* C356 and the *in vivo* passaged C356P were indistinguishable (experiment 4; figure 3b). Which of the many possible factors (Ahmed et al., 2002; Hanel et al., 2008) is responsible for the difference in *in vivo* colonization potential between these strains is not identified. The identical embryonic dose response curves indicate that additional factors influence the establishment of colonization in *in vivo* experiments.

We explored the colonization characteristics of *C. jejuni* in 16 days old chicken embryos. Based on the colonization levels in the embryonic intestines, we conclude that *Campylobacter* growth conditions in chicken embryos are good. The observed quantitative dose response relation indicates that the model might be suitable to estimate the number of viable *Campylobacter* cells in inoculums, which might be useful for assessing *Campylobacter* contamination levels.

Qualitative dose response modeling showed that the model is highly sensitive, both for strains with a high and low *in vivo* colonization potential.

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Chapter 7

General discussion

Campylobacter spp. are generally regarded as the most common cause of bacterial foodborne disease worldwide, and poultry is generally considered an important source of foodborne campylobacteriosis, with mishandling resulting in cross-contamination of other foods as the main route of human infection (Luber, 2009). Interventions aimed at reducing poultry-related exposure of *Campylobacter* to humans are therefore likely to reduce the incidence of human campylobacteriosis and the subsequent disease burden.

Measures to reduce human exposure can be applied in kitchens, slaughterhouses and poultry farms. The development and optimization of control strategies requires knowledge of the epidemiology of *Campylobacter* both in humans and in poultry. The research in this thesis focused on the epidemiology of *Campylobacter* in the primary sector, i.e. the broiler flock.

In the process of *Campylobacter* colonization in broiler flocks five steps at which control strategies can be targeted, can be distinguished:

- introduction into the broiler house;
- exposure of broilers;
- first colonization of an exposed broiler;
- transmission within the flock;
- reduction of colonization levels.

Using the methods and newly acquired information described in this thesis, current knowledge on interventions and methods to evaluate interventions are discussed for each of the above steps.

INTRODUCTION INTO THE BROILER HOUSE

Campylobacter spp. are able to colonize the intestines of various animal species (Newell et al., 2003), and are therefore widespread in the environment. This implies that *Campylobacter* can be introduced into a broiler house via many different routes, either directly from defecating animals or passively via fecal material attached to feed particles, equipment, clothes etc. or other animals, not necessarily colonized. Field studies indicated that *Campylobacter* was introduced via flies (Guerin et al., 2008; Hald et al., 2008; Hald et al., 2007), drinking water (Arsenault et al., 2007; Ellis-Iversen et al., 2009), farm workers (Cardinale et al., 2004; Evans et al., 2000; Gibbens et al., 2001; Hald et al., 2000; McDowell et al., 2008), and equipment (Allen et al., 2008; Ramabu et al., 2004). The variety of sources of introduction makes it extremely difficult to develop and maintain an adequate and sustainable control program on farm level, because it is unknown which are the most important ones.

In addition to the horizontal transmission routes above, persistence of *Campylobacter* in the broiler house and vertical transmission from parent stock might be expected to contribute to broiler colonization. There is however no evidence that *Campylobacter* spp. shed by colonized broilers during a previous production cycle cause colonization in subsequent flocks (Shreeve et al., 2002), suggesting that routine broiler house cleansing and disinfection procedures are adequate to eliminate *Campylobacter*. There is also no evidence that introduction of *Campylobacter* via day-old chicks is a significant source of broiler colonization (Callicott et al., 2006; Jacobs-Reitsma et al., 1995; Newell et al., 2003).

In chapter 3 the first colonized flock was >3 weeks of age, and also in other studies a ‘lag phase’ was described (Bull et al., 2006; Gregory et al., 1997; Jacobs-Reitsma et al., 1995; Ring et al., 2005). This might be caused by limited introduction of *Campylobacter* in the first few weeks. It is imaginable that some factors are increasingly contributing to *Campylobacter* introduction as flocks grow older. The flow of ventilation air increases over time, which may increase the risk of influx of flies (Hald et al., 2008). Non-compliance with hygiene measures at dead birds removal might also pose a risk, as demonstrated for *Salmonella* colonization in broilers (Gradel et al., 2003), and is also likely to increase over time as the volume of dead birds increases. Current knowledge on possible sources of introduction can however not explain why introduction would be limited to the second half of the rearing period. It is therefore more likely that other factors than absence of introduction cause the lag phase.

Although many epidemiological studies have been carried out to identify risk factors and sources of introduction for *Campylobacter* (Adkin et al., 2006), the quantitative role of various routes is still unclear. Most observational studies based the colonization status of the flock on samples collected at the end of the production period, shortly before slaughtering. The quantification of risk factors by this method is hampered by the presence of multiple introduction routes during the entire rearing period. Moreover, exact routes and the moment of introduction remain unknown, which makes it impossible to pinpoint a certain source.

For further quantification of the contributions of the various potential introduction routes to broiler colonization, it would be useful to know this moment of introduction. Estimation of this parameter requires knowledge on the rate at which the bacteria colonizes the flock. This type of information has been used successfully in tracing studies during epidemics with classical swine fever (Stegeman et al., 1999), and avian influenza (Bos et al., 2009). These studies used the prevalence of seropositive or diseased animals. In combination with a mathematical model that describes the population dynamics of the pathogens in the population, they could give a rather precise estimate of the moment of introduction. In chapters 2 and 3, it was illustrated that frequent sampling of individual broilers can provide accurate estimates of β, which were subsequently used to estimate a more exact moment of colonization of the first bird, i.e. t_0. Such a narrow estimate of the moment of introduction is useful to relate *Campylobacter* introduction to events, e.g. farm visits, or risk factors, e.g. change of feed composition, and is therefore helpful to identify introduction routes, which in turn might contribute to the development or optimization of intervention measures.

Field studies have been assessed to determine if interventions successfully delayed the onset of detectable colonization. Hygiene measures in the anteroom, including a clear demarcation zone, washing hands, and changing boots (Gibbens et al., 2001; van de Giessen et al., 1996) and the use of fly screens (Hald et al., 2007) have shown to delay detection of flock colonization. In these studies, the flock colonization status was assessed once a week, and the age till detection was recorded. Alternatively, based on the knowledge on β provided in chapter 2 and 3, appropriate sampling schemes can be chosen that allow for the estimating of a more accurate estimate of the delaying effect of an intervention, i.e. t_0.

EXPOSURE OF BROILERS

Between incursion and exposure, the bacteria should be able to survive in the environment. High temperatures and dry conditions reduce survival of *Campylobacter* (Brandl et al., 2004; Line, 2006). Because these conditions are typical for the beginning of the rearing period (Dawkins et al., 2004; Radoń et al., 2004), this might explain the observed absence of colonization in the first half of the rearing period (chapter 3). These observations might also give lead to the development of control measures.

It is not exactly clear what the conditions are that optimize survival of *Campylobacter* in the broiler house. Up to now, most studies on inactivation of the bacteria have been done in a laboratory setting. Nevertheless, intervention measures have been developed to reduce *Campylobacter* survival in the field. To promote inactivation of *Campylobacter* throughout the rearing period, litter acidification has been suggested (Line et al., 2006). Due to the ongoing accumulation of feces, such treatment might however not be effective throughout the rearing period (Ruiz et al., 2008).

Interventions have been suggested for specific situations: after partial depopulation, when *Campylobacter* might have been introduced by contaminated transport crates or catching crew (Allen et al., 2008), litter in the depopulated area in a house could be left to dry, resulting in inactivation of introduced bacteria (Rosenquist et al., 2007). Then, to prevent exposure of remaining birds in the meanwhile, access of these birds to the depopulated area should be prevented temporarily. The effectiveness of such a measure has not been studied, and its feasibility is debatable, as the aim of thinning is to give more space to the remaining birds in the flock.

When evaluating interventions, survival of *Campylobacter* could be measured under experimental conditions. To determine the survival, a diagnostic technique that detects all viable *Campylobacter* cells would be preferred. Standard microbiological techniques might fail to detect viable *Campylobacter* cells as exposure to environmental stress, e.g. acid (Chaveerach et al., 2003) or a lack of nutrients (Cappelier et al., 1999; Medema et al., 1992), might result in viable but nonculturable (VBNC) *Campylobacter* cells. A more sensitive test was suggested by Cappelier et al. (1999) and Talibart et al. (2000) who resuscitated VBNC *Campylobacter* in embryonated chicken eggs. The chicken embryo colonization model described in chapter 6 seems a sensitive detection method that might be

useful for the detection of both fully viable and VBNC cells when evaluating interventions that aim to inactivate *Campylobacter*. Further research on the chicken embryo colonization model is required to determine how environmental samples, which may contain microbial contaminants, can be analyzed without affecting the outcome of the test or survival of the embryo.

FIRST COLONIZATION OF AN EXPOSED BROILER

The probability that exposure of a broiler results in colonization is determined by the number of ingested viable *Campylobacter* cells or 'dose', and the probability for each single bacterium in that dose to colonize the gut (Teunis et al., 2000). The latter probability depends on factors related to the bacteria and factors related to the host, i.e. susceptibility of the broiler.

The absence of colonization in the first half of the rearing period as observed in chapter 3 might be caused by low doses of bacteria and/or low susceptibility of broilers during this period. The effect of ingested dose on colonization success was studied in chapter 4. In two experiments with individually housed birds, the inoculation dose that colonizes 50% of inoculated broilers (CD_{50}) was determined. Indeed, inoculation with a low dose resulted in a lower percentage of colonized birds, which might be one of the explanations for the lag phase.

The susceptibility of the bird may affect the success of colonization. Feed composition, which is commonly adjusted several times throughout the rearing period, has been suggested to affect susceptibility (Wagenaar et al., 2008), but has however not been studied.

One factor affecting the host's susceptibility is age. It has been shown that day-old Specified Pathogen Free (SPF) chicks are more susceptible for colonization than 2-week-old SPF chicks (Knudsen et al., 2006). This difference is possibly associated with the developing gut flora (Lu et al., 2003), but might also relate to the immature immune system of young broilers (Ask et al., 2007).

Another factor contributing to a lower susceptibility of the young commercial broiler is the presence of maternally derived antibodies (MDA) during the first two weeks of the bird's life (Sahin et al., 2001). These MDA originate from the hen in the parent flocks which are commonly *Campylobacter*-positive and seropositive (Callicott et al., 2006; Sahin et al., 2001). Shanker et al. (1988) showed that the

susceptibility for 2-day-old and 2-week-old commercial chickens was similar, which seems suggestive for a lack of effect of MDA. Considering the above study of Knudsen et al. (2006), it can however be hypothesized that, under commercial conditions, the higher susceptibility of MDA-negative day-old chicks is compensated by an effect of MDA. The exact mechanism remains however to be elucidated.

Several studies have been carried out on the development of interventions that reduce the susceptibility of the bird by stimulating specific immunity or by supplementation of components that promote the inactivation of *Campylobacter* within the gastro-intestinal tract. Development often starts with the evaluation of *in vitro* anti-*Campylobacter* activity. Such activity has been demonstrated for bacteriophages (Wagenaar et al., 2005), *Campylobacter*-specific antibodies (Sahin et al., 2003), carboxyil acid (Chaveerach et al., 2002), fatty acids (Thormar et al., 2006) and bacteriocins (Stern et al., 2005; Stern et al., 2006). *In vitro* exposure of *Campylobacter* to these components, however, poorly mimics the situation in the avian gut. The embryo colonization model described in chapter 6 may provide the complex structure of the avian gut, possibly offering improved conditions for studying interaction between *Campylobacter*, the intervening component and the host gastrointestinal tract. In a pilot study (van Gerwe et al., unpublished data), a combination of two Medium Chain Fatty Acids (MCFA), was injected into the amniotic fluid, directly followed by injection with *C. jejuni*. Though effective *in vitro*, none of the concentrations successfully prevented embryonic colonization, which might have been due to the strong buffering capacity of albumin in the amnion fluid (Yoshizaki et al., 2002), causing a shift of MCFA towards its non-active dissociated form (van der Wielen et al., 2000). Although proof of principle for intervention studies has so far not been illustrated, this model is potentially useful to study pathogenesis, the effect of changes in bacterial characteristics, and to assess the efficacy of candidate intervening components.

Studying the effect of an intervention *in vivo* can be considered the final evaluation step. Studies on change in susceptibility of the bird have predominantly focused on the effect of competitive exclusion (CE) (Lin, 2009) and vaccination (de Zoete et al., 2007). CE is highly successful for *Salmonella* spp. (Mead, 2000), but the efficacy for *Campylobacter* spp. is limited and seems unpredictable (Lin, 2009; Wagenaar et al., 2008). Moreover, difficulties with the complete identification of included species, required for safety of the poultry flock and human consumers, limits the development of commercial CE products (Wagner, 2006). The effect of vaccination against *Campylobacter* has also been investigated by challenge

inoculation (Wyszynska et al., 2004; Ziprin et al., 2002), and exposure to seeder chickens (Rice et al., 1997). In these studies no difference in the number of colonized individuals was observed, which might have been caused by the high exposure doses used.

A more sensitive method is to determine the effect of interventions on CD_{50} (Line et al., 2008), as in chapter 4. In chapter 4, MCFA feed-supplementation was shown to increase CD_{50} two hundred fold while improving body weight gain. Analysis of data from a study on the effect of Short Chain Fatty Acids (Heres et al., 2004) showed a similar increase in CD_{50}, but broiler performance was negatively affected. In commercial broiler production, this is undesirable, and therefore 1% MCFA feed-supplementation is more likely to be implemented in the field.

The *in vivo* colonization model in chapter 4 showed comparable dose response curves for the two experiments conducted. In future experiments with a similar experimental design, inoculation with a quantified dose will result in predictable rates of colonized broilers. This knowledge can be employed for the qualitative evaluation of interventions in single dose experiments, similar to the above mentioned vaccine studies. For low and high inoculation doses, difference in colonization rate in control and treatment birds will be limited. For intermediate doses, large differences can be expected if the treatment has an effect on CD_{50} similar to, or greater than, the MCFA treatment in chapter 4. To detect such large difference in colonization rate, only limited numbers of broilers per treatment group are required. In conclusion, inoculation with a single, intermediate dose of *Campylobacter* can be attractive for the screening of multiple candidate intervening components or various concentrations of a single component.

Under field conditions, various factors might influence the effect of an intervention. Implementation of an apparently effective intervention, like the MCFA feed-supplementation in chapter 4, does therefore not necessarily result in a reduction of the number of positive flocks at slaughtering. As previously discussed for the prevention of introduction, assessing whether an intervention delays the onset of colonization is therefore the most appropriate evaluation method in the field.

TRANSMISSION WITHIN THE FLOCK

After an initial latent period, defined as the period between exposure and start of shedding, *Campylobacter* spp. are shed by the first colonized broiler. The latent period is rather short, generally 1 to 3 days, as shown in several experimental studies (Heres et al., 2004; Young et al., 1999), including the studies described in chapters 2 and 4.

Once contact birds are exposed to doses that induce colonization, and shed bacteria in the feces, transmission may occur. Whether or not this happens, depends on infectiousness of shedding birds, susceptibility of contact-exposed birds and the contact rate between the two types (Diekmann et al., 2000). Transmission can be quantified by transmission parameter β (Diekmann et al., 2000; Keeling et al., 2008), for experiments (chapter 2) and for transmission in the field (chapter 3). The application of parameter β in epidemiological studies has been described in these chapters and in previous sections in this general discussion.

It was hypothesized in the study in chapter 2 that exposure to low doses of *Campylobacter* might delay transmission by extending both the latent period in the first colonized bird, and the time between start of shedding and start of transmission. In an experimental study with various doses, the latent period and cecal colonization levels were estimated (van Gerwe et al., unpublished data). Three low dose groups (approximately 1, 2, and 3 ^{10}Log cfu/bird), each consisting of 35 individually housed chicks, and a high dose group (approximately 7 ^{10}Log cfu/bird; n=10) were inoculated with *C. jejuni* C356. Low inoculation doses did not affect the latent period and the cecal *Campylobacter* colonization levels, suggesting that the time till start of transmission, or 'delay time', is independent of exposure dose. These findings indicate that replication of *Campylobacter* within the ceca occurs rapidly, and small differences in initial doses are overwhelmed by the amount of bacteria in the ceca within the few hours in which the replication occurs.

Although experiments are always rather artificial, an advantage is that the exact moment of exposure is known, in contrast to field studies. Under commercial conditions, the moment of colonization and the number of initially colonized broilers cannot be determined. Therefore, it is very difficult to determine if a delay time is likely to exist in the field, although there are no indications that it is absent. In the transmission experiments (chapter 2), estimated delay times varied from 2.4 to 7.1 days. Although the inoculated broilers (seeders) had considerable latent periods, latency could not explain observed delay times to their full extent.

The estimated delay times resulted from modeling of the experimental data using a Susceptible-Infectious (SI) model (Diekmann et al., 2000; Keeling et al., 2008). This mathematical model ignores the existence of a latent period (E), and assumes colonization to persist throughout the rearing period. Fitting other transmission models, e.g. a SEI-model, with a latent period, or a SIS-model, with recovery from colonization resulting in a susceptible individual, did also not explain the observed delay time (Conlan et al., 2007).

In a SI-model the transmission parameter β is assumed to be constant during the outbreak (Diekmann et al., 2000; Keeling et al., 2008). If in reality β increases over time, SI modeling as applied in chapters 2 and 3 is likely to result in a delay time. The delay time might represent a period in which environmental contamination is built up resulting in indirect transmission, e.g. via drinking water, in a subsequent phase. By choosing the SI-model, one implicitly assumes that the transmission of *Campylobacter* between individuals can be approximated by a direct transmission process. Even if transmission is predominantly a result from environmental contamination that requires such a build up phase, transmission might be modeled correctly with a SI-model, as long as a delay time is fitted to the data, and transmission in the build-up phase is negligible.

The implicit assumption of the SI-model that the rate at which an infectious bird causes colonization in susceptible individuals is independent on the numbers of birds might however be violated when transmission occurs mainly indirect. When, e.g. high doses of *Campylobacter* spp. would spread via the drinking water system, which typically consists of a pipe-line over the full length of the house, in large flocks more broilers might get colonized per infectious individual than in small flocks. The difference in the estimated β in chapter 2 and 3 might therefore be related to difference in flock sizes, which were on average 20,000 in the commercial flocks (chapter 3) and 400 in the experiments described in chapter 2. If estimation of β would indeed be affected by indirect transmission and flock size, transmission of *Campylobacter* should preferably be quantified with models that take into account environmental contamination, instead of the SI-model used in this thesis.

The β estimate for the transmission experiments with day-old broilers in chapter 2 suggests that detectable flock colonization (10% prevalence) can be reached within 7 to 8 days once transmission starts. The values for transmission rate parameter β observed in commercial flocks (>3 weeks of age) suggest that detectable flock colonization is established within 2.6 to 4.3 days (chapter 3). Even when delay

times estimated in chapter 2 are added, both estimates suggest that the commonly observed lack of detectable colonization in the first 2 to 3 weeks (Bull et al., 2006; Gregory et al., 1997; Jacobs-Reitsma et al., 1995; Ring et al., 2005) is predominantly a result of a lack of colonization rather than the time required for detectable colonization to establish.

Transmission in commercial broiler flocks, which was described by the field estimate for β (chapter 3), indicates that even late introduction of *Campylobacter*, e.g. one week before depopulation, can result in fully colonized flocks at slaughtering. Reliable prediction of the *Campylobacter* status at slaughtering therefore requires flock sampling shortly before scheduled slaughtering, and even then of course the flock might have been partly colonized but misclassified as 'negative', like in all tests carried out on flock level.

Interventions that reduce survival of *Campylobacter* in the environment, or reduce the susceptibility of the broiler, might also affect transmission. Additionally, the amount of shed bacteria, and the contact structure between broilers are likely to affect transmission. Considering the transmission rate of *Campylobacter* in the field (chapter 3), interventions should reduce transmission considerably to be effective. Consequently, such measures may be able to reduce the number of colonized broilers at slaughtering only in situations where late introductions of *Campylobacter* spp. occur.

The variation in transmission rates seems to vary less in experimental flocks compared to commercial flocks (chapter 2 and 3), which means that experiments can be suitable for evaluation of the effect of measures on the reduction of transmission, like performed for *Mycoplasma gallisepticum* vaccination of chicken (Feberwee et al., 2006a; Feberwee et al., 2006b). Considering the above mentioned uncertainty about the appropriate modeling of transmission, accurate quantification of the effect of an intervention might eventually require evaluation studies in the field.

REDUCTION OF COLONIZATION LEVELS

The above sections of the general discussion describe the state of the art with respect to the establishment of *Campylobacter* colonization in broiler flocks, the contribution of the research in this thesis, and possible intervention measures that aim to prevent *Campylobacter* colonization in broilers. Despite all effort,

Campylobacter is still common in the poultry industry. As an alternative to reducing the number of colonized flocks, reduction of colonization levels at slaughtering could be considered.

Highest cecal colonization levels are observed 5 days after onset of colonization (Wagenaar et al., 2008). Although a slight decrease in colonization level might occur afterwards, the majority of broilers will shed *Campylobacter* in concentrations above 6 ^{10}Log cfu/g feces for the remaining rearing period (El-Shibiny et al., 2005; Knudsen et al., 2006). Interventions might be implemented shortly before slaughtering to reduce colonization levels. To predict the efficacy of an intervention that reduces colonization levels, knowledge on the relation between colonization levels and carcass contamination levels is required. Most studies on the relation between intestinal colonization levels and carcass contamination did not detect a correlation (Allen et al., 2007; Nauta et al., 2009; Stern et al., 2003), and the correlation between crop colonization levels and carcass contamination remains unclear.

In chapter 5, the observed crop colonization levels confirmed that crop content might contribute to carcass contamination. A correlation between crop and cecal colonization levels was lacking. Consequently, the above mentioned lack of correlation between intestinal and carcass levels cannot be considered indicative for the relation between crop and carcass levels. It is therefore recommended to included crop colonization levels in future studies on cross-contamination.

Once the quantitative relation between specified bird colonization levels and carcass contamination is revealed, the desired reduction in these colonization levels can be determined. Subsequently, interventions that successfully reduce carcass contamination can be developed and evaluated. Additionally, it is recommended to study the effect of reduced carcass contamination levels on the incidence of *Campylobacter* infections in humans.

CONCLUDING REMARKS

Campylobacter colonization in commercial broiler flocks is rarely detected in the first few weeks of the rearing period. The observed lack of *Campylobacter* in the study population described in chapter 3, combined with estimated transmission rates (chapter 2 and 3), suggests that broilers are less exposed to *Campylobacter* during the first few weeks. Implementation of interventions should therefore be

focused on the second half of the rearing period. The studies in chapter 2 and 3 also provided tools to carry out field studies to elucidate risk factors or sources of introduction more specifically than has been done during the last years. Sample size and sample frequency required to estimate the moment of colonization of the first bird (t_0) have been calculated. Accurate estimates of t_0 narrow the period in which the bacteria might have been introduced, which can help to identify the correct source of introduction.

If introduction cannot be prevented, reduction of successful colonization of the first bird could be achieved by a reduction of the susceptibility of broilers, e.g. by feed supplementation (chapter 4). Once colonized, shedding does not seem to be affected, but the CD_{50} is substantially increased, resulting in a higher dose needed for establishment of the colonization. If this amount of bacteria is not present, the flock may remain negative.

Reducing susceptibility contributes to the prevention of broiler colonization. The estimated dose response curves indicate that the efficacy of an intervention might be limited for high exposure doses (chapter 4), but might be effective when exposure doses are low. If birds are exposed to high doses in the field, decreasing susceptibility might therefore be inadequate and reduction of *Campylobacter* exposure would be required. To achieve this, current knowledge on introduction routes could be used, and remaining introduction routes may be traced by estimation of t_0 (chapter 3). In addition, interventions that limit survival of introduced *Campylobacter* can be evaluated using the sensitive embryo colonization model described in chapter 6.

If eventually colonization of an exposed bird cannot be prevented, interventions might limit the number of colonized broilers within a flock at slaughter age by reducing transmission. Considering the observed field transmission rate (chapter 3), such interventions are most likely to be effective for late introductions only.

In colonized flocks, interventions that reduce colonization levels in the digestive tract at slaughtering might reduce human exposure. The findings in chapter 5 indicate that more research is however required to determine if the crop contents contributes significantly to carcass contamination.

Various methods and models have been used, evaluated and improved in this thesis. Depending on the aim of future studies on pathogenesis or the aim of control measures that are developed, the models described in the various chapters

could prove useful. The transmission model might be suitable to study factors that affect transmission and to identify introduction routes by estimating t_0. The *in vivo* dose response and colonization level model might be suitable to evaluate interventions measures that reduce susceptibility and colonization levels, respectively. The embryo model might be used as more sensitive test to detect *Campylobacter* strains or to study pathogenesis. In addition, future research could reveal if the embryo colonization model is useful for the screening of potential intervening components.

It is unlikely that *Campylobacter* infections in humans can be prevented by a single measure in primary production. Improvement of on-farm hygiene and biosecurity will definitely contribute to a lower exposure in broilers. To further reduce the number of colonized flocks additional measures like the use of MCFA are needed. As it is possible to determine the moment of *Campylobacter* introduction more precisely, analysis of risk factors and therewith intervention methods can be more directed. By combining previously identified preventive measures, interventions described in this thesis, and risk factors to be identified by use of the more specified moment of introduction, a reduction of poultry-related *Campylobacter* exposure in humans may be obtained.

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Summary

CAMPYLOBACTERIOSIS IN HUMANS

Campylobacter spp., in particular *C. jejuni* and *C. coli*, are considered to be a major cause of diarrheal illness in humans and are generally regarded as the most common cause of bacterial foodborne disease worldwide. The onset of campylobacteriosis is often acute, with abdominal cramps shortly followed by diarrhea. The majority of patients recover within a week after onset of the symptoms, but some develop complications after the acute phase. The incidence rate of *Campylobacter*-associated illnesses has been estimated at approximately 900 cases per 100,000 inhabitants *per annum* in England and the Netherlands.

Although its contribution cannot be quantified precisely, poultry is generally considered the most important source of foodborne campylobacteriosis. Mishandling of poultry meat in the consumers kitchen resulting in cross-contamination of other foods is considered the most important route of infection. Interventions aimed at reducing poultry-related exposure of *Campylobacter* to humans are therefore likely to reduce the incidence rate and the disease burden of campylobacteriosis.

CAMPYLOBACTER IN POULTRY

Campylobacter does not cause clinical signs or pathological lesions in poultry. Within the poultry industry, broilers account for the largest poultry meat market worldwide. Field studies have provided knowledge on risk factors for colonization of commercial broilers, but the exact routes of transmission remain largely unknown. To identify these routes and develop specific interventions, information on the moment of introduction would be useful. Because it is unfeasible to detect the first bird that is colonized, knowledge on the dynamics of *Campylobacter* colonization within a flock is required to determine the moment of introduction. Therefore, in **chapter 2** transmission of *Campylobacter* among broilers was studied in experimentally exposed groups of broilers. In the four experiments, transmission started 2.4 to 7.1 days after inoculation. The reason for this delay in transmission was not revealed in this study, and it remains unclear if it is a result of the experimental conditions or might also occur under field conditions. The estimated transmission rate parameter (β) indicated that once transmission starts, each infectious bird will infect on average 1.04 new broilers per day. This would imply that in a flock of 20,000 broilers the prevalence of *Campylobacter* would increase to 95% within 12.5 days after start of transmission.

As experimental conditions differ from the field situation, the transmission rate in commercial flocks might differ from the experimentally derived transmission rate, and might vary between flocks. Therefore, in **chapter 3**, sampling data for 42 *Campylobacter jejuni*-colonized flocks were used to estimate the distribution of β under field conditions. The distribution of β was estimated at 2.37 ± 0.295 newly colonized birds per infectious bird per day. This implies that in the study population colonized flocks, which on average consisted of 20,000 broilers, had an increase in within-flock prevalence to 95% within 4.4 to 7.2 days after start of transmission. Consequently, it can be concluded that even late introductions of *Campylobacter*, e.g. one week before depopulation, might result in fully colonized flocks at slaughtering. Additionally, the moment that the first bird in a flock was colonized (t_0) was estimated using Bayesian analysis. In all colonized flocks in the study t_0 was estimated to be over 21 days of age. Although it could not be determined if introduction of bacteria did occur in the first 3 weeks, the results strongly suggest that the probability for commercial broilers flocks to become colonized is reduced in the first few weeks compared to the second half of the rearing period. Therefore, the desired intensification of interventions aimed at prevention of introduction of and subsequent colonization by *Campylobacter* might better focus on the second half of the rearing period.

Analysis of simulated outbreaks showed that t_0 can be estimated with an accuracy of approximately 3 days when flocks are sampled every 3rd day. The method described in **chapter 3** can therefore be considered a promising method for further studies on the exact routes of *Campylobacter* introduction.

Despite efforts to prevent *Campylobacter* introduction under field conditions, reduction of the susceptibility of broilers for colonization remains important. This might be achieved by feed supplementation. Therefore, in **chapter 4**, the effect of feed supplementation with Medium Chain Fatty Acids (MCFA) on the susceptibility for *Campylobacter* was studied in individually housed commercial broilers by determining the relation between the *Campylobacter* inoculation dose and the subsequent likelihood of colonization to occur. The dose response curve of broilers that were provided MCFA supplemented feed was shifted to the right, indicating that they required a 200 times higher inoculation dose to become colonized than non-supplemented broilers. MCFA feed supplementation might therefore be a helpful tool for the reduction of susceptibility in broilers. Because the dose response curves indicated that the effect of the intervention might be limited for high exposure doses, and the *Campylobacter* exposure dose that broilers experience in the field is unknown, field trials are necessary to determine to what

extent MCFA supplementation reduces *Campylobacter* colonization under field conditions.

If eventually flock colonization cannot be prevented, interventions that reduce colonization levels in the digestive tract at slaughtering might reduce carcass contamination levels and subsequently human exposure. Both the upper part of the digestive tract, in particular the crop, and the intestines might cause cross-contamination of the carcass at evisceration. Therefore, in **chapter 5** the effect of drinking water supplementation with volatile fatty acids (VFA) on *Campylobacter* levels was assessed in crop and ceca of *Campylobacter*-colonized broilers. In addition, the correlation between crop and cecal colonization levels was determined to assess whether colonization levels in a sample from one organ were predictive for colonization levels of the sample from the other organ. VFA supplementation did not result in a significant reduction of colonization levels neither in the crop nor in the ceca, and no correlation between crop and cecal *Campylobacter* levels was detected.

Colonization properties of *Campylobacter* are usually studied in chickens. Alternatively, a colonization model in chicken embryos might resemble the micro-environmental conditions in young chickens, and might allow for the evaluation of *Campylobacter* colonization potential and bacterium-host interaction in absence of gut flora. To assess the value of an embryo colonization model, in **chapter 6** colonization characteristics in intestines were studied in embryonated eggs at 16 days of incubation. Duodenum and cecum showed a positive correlation between inoculation dose and colonization level at 1 day post inoculation (PI), and maximal colonization levels reached 2 days PI remained constant till 3 days PI. The observed colonization levels suggested that the embryo model provides conditions favoring a steady growth of *Campylobacter* with colonization levels in cloacal content comparable to colonization in live birds. Furthermore, assessment of the analytical sensitivity, defined as the probability of colonization following inoculation of one cfu *Campylobacter* was estimated 0.35 and 0.7 in the two experiments conducted. Both values are orders of magnitude higher than the average value determined in live birds in chapter 4. In conclusion, the embryo model is a sensitive tool for the detection of *Campylobacter*.

CONCLUDING REMARKS

Various methods and models have been used, evaluated and improved in this thesis. Depending on the aim of future studies on pathogenesis or the aim of control measures that are developed, the models described in the various chapters could prove useful. The transmission model (**chapter 2 and 3**) might be suitable to study factors that affect transmission and to identify introduction routes by estimating t_0. The dose response model (**chapter 4**) and the colonization level model (**chapter 5**) might be suitable to evaluate interventions measures that reduce susceptibility and colonization levels, respectively. The embryo model (**chapter 6**) might be used as more sensitive test to detect *Campylobacter* strains or to study pathogenesis.

It is unlikely that poultry-related *Campylobacter* infections in humans can be prevented by a single preventive measure implemented in primary production. Improvement of on-farm biosecurity will definitely contribute to a lower exposure in broilers. To further reduce the number of colonized flocks additional measures like the use of MCFA are most likely needed. As it is possible to determine the moment of *Campylobacter* introduction more precisely, analysis of risk factors and therewith intervention methods can be more directed. By combining previously identified preventive measures, interventions described in this thesis, and risk factors to be identified by use of more specified moments of introduction, a reduction of poultry related *Campylobacter* exposure in humans may be obtained.

Samenvatting

HUMANE CAMPYLOBACTERIOSE

Campylobacter spp., in het bijzonder *C. jejuni* and *C. coli*, zijn belangrijke veroorzakers van diarree bij mensen and worden wereldwijd beschouwd als de meest voorkomende oorzaak van bacteriële voedselinfecties. Campylobacteriose wordt gekenmerkt door acute buikkramp, gevolgd door diarree. De meeste patiënten herstellen binnen een week na aanvang van de klachten, maar sommigen ontwikkelen complicaties na de acute fase. De incidentie van campylobacteriose in Engeland en Nederland wordt geschat op ongeveer 900 gevallen per 100.000 inwoners per jaar.

Hoewel de exacte bijdrage niet bekend is, wordt pluimvee beschouwd als de belangrijkste bron van *Campylobacter* voedselinfecties. Kruiscontaminatie van ander voedsel tijdens de bereiding van besmet pluimveevlees in de keuken wordt beschouwd als de belangrijkste infectieroute. Maatregelen die de bedoeling hebben om pluimveegerelateerde humane blootstelling te beperken, leiden daarom waarschijnlijk tot een reductie van de incidentie en ziektelast van campylobacteriose.

CAMPYLOBACTER IN PLUIMVEE

Campylobacter veroorzaakt in pluimvee geen ziekteverschijnselen of pathologische afwijkingen. Het overgrote deel van de mondiale pluimveevleesproductie is afkomstig van de vleeskuikenindustrie. Daarom heeft het onderzoek zich vooral gericht op deze sector. Veldstudies hebben ons inzicht verschaft in de risicofactoren voor *Campylobacter*-kolonisatie in commercieel gehouden vleeskuikens. Hoe besmettingen tot stand komen is echter nauwelijks bekend. Om de introductieroutes te kunnen identificeren en ten behoeve van de ontwikkeling van specifieke maatregelen is informatie over het tijdstip van introductie van belang. Omdat het onmogelijk is om het eerst besmette vleeskuiken te detecteren, is kennis van de verspreiding van *Campylobacter* in een koppel nodig om het tijdstip van introductie retrospectief te kunnen schatten. Daarom is in **hoofdstuk 2** transmissie van *Campylobacter* tussen vleeskuikens bestudeerd in experimenteel blootgestelde groepen vleeskuikens. In de vier experimenten startte de verspreiding enigszins vertraagd, namelijk tussen 2.4 tot 7.1 dagen na inoculatie. De reden voor deze vertraging werd niet opgehelderd in deze studie en het blijft dan ook onduidelijk of de vertraging het gevolg is van de experimentele omstandigheden of ook onder praktijkomstandigheden zou kunnen voorkomen. De

geschatte transmissieparameter (β) voorspelt dat zodra de transmissie van start gaat, elke infectieuze kip gemiddeld 1,04 ander kippen zal besmetten per dag. Dit betekent dat de prevalentie van *Campylobacter* in een koppel van 20.000 dieren binnen 12,5 dagen zal stijgen tot 95%.

Omdat experimentele omstandigheden verschillen van praktijkomstandigheden zou de transmissiesnelheid in commerciële vleeskuikenkoppels kunnen verschillen van de experimenteel verkregen waarde. Bovendien zou ze kunnen variëren tussen koppels. Daarom zijn in **hoofdstuk 3** monsternamegegevens van 42 *Campylobacter*-positieve koppels gebruikt om de distributie van β onder praktijkomstandigheden te bepalen. Deze werd geschat op 2.37 ± 0.295 nieuwe besmettingen per infectieuze kip per dag. Dit betekent dat in gekoloniseerde koppels (die gemiddeld bestonden uit 20.000 vleeskuikens) de prevalentie van gekoloniseerde vleeskuikens tussen 4.4 tot 7.2 dagen na de start steeg tot 95%. Er kan dan ook geconcludeerd worden dat zelfs late introducties van *Campylobacter*, b.v. één week voor het slachten, kunnen resulteren in volledig gekoloniseerde koppels op het moment van slachten. Ook was het moment van introductie geschat middels een Bayesiaanse analyse. Alle koppels raakten gekoloniseerd na 3 weken leeftijd. Hoewel niet kon worden bepaald of er in de eerste drie weken introductie had plaatsgevonden, suggereert deze bevinding dat de kans voor commerciële vleeskuikenkoppels om besmet te raken in de eerste paar levensweken lager is dan in de tweede helft van de productieperiode. Gewenste verbeteringen van interventiemaatregelen gericht op de preventie van introductie zouden daarom waarschijnlijk het best gericht kunnen worden op de tweede helft van de productieperiode.

Analyse van gesimuleerde uitbraken toonden bovendien aan dat t_0 geschat kan worden met een nauwkeurigheid van 3 dagen als koppels elke derde dag bemonsterd worden. De methode zoals beschreven in **hoofdstuk 3** kan dan ook beschouwd worden als een veelbelovende methode voor toekomstig onderzoek naar de exacte introductieroutes van *Campylobacter*.

Ondanks inspanningen om *Campylobacter* introductie in vleeskuikenkoppels te voorkomen, blijft het belangrijk om de gevoeligheid van vleeskuikens voor kolonisatie met *Campylobacter* te verlagen. Dit zou o.a. gerealiseerd kunnen worden middels voersupplementen. Daarom werd in **hoofdstuk 4** het effect van supplementatie met middellange keten vetzuren (MCFA) op de gevoeligheid voor *Campylobacter* bestudeerd. In individueel gehuisveste commerciële vleeskuikens werd de relatie tussen de *Campylobacter* inoculatiedosis en de daaruit

voortvloeiende kans dat een kuiken gekoloniseerd raakte gekwantificeerd. De benodigde dosis om *Campylobacter*-kolonisatie te veroorzaken bleek 200 maal groter in MCFA gesupplementeerde kuikens dan in niet gesupplementeerde vleeskuikens. MCFA voersupplementatie kan dan ook beschouwd worden als een veelbelovend middel voor de verlaging van de gevoeligheid van vleeskuikens. Aangezien de dosis response kromme toonde dat het effect van de interventie beperkt kan zijn voor hoge blootstellingniveaus, en de blootstellingniveaus onder veldomstandigheden onbekend is, zijn veldstudies noodzakelijk om te bepalen tot op welke hoogte MCFA supplementatie het aantal *Campylobacter*-positieve koppels werkelijk verlaagd.

Als uiteindelijk kolonisatie van het koppel vleeskuikens niet kan worden voorkomen, kunnen interventies die de kolonisatieniveaus in het spijsverteringsstelsel van het vleeskuikens verlagen resulteren in verlaagde karkas besmettingniveaus en vervolgens verlaagde blootstelling bij de mens. Zowel het proximale deel van het spijsverteringsstelsel, in het bijzonder de krop, als de darmen kunnen kruiscontaminatie van het karkas veroorzaken ten tijde van evisceratie. Daarom is in **hoofdstuk 5** het effect van drinkwater supplementatie met vluchtige vetzuren op *Campylobacter* niveaus bestudeerd in krop en blinde darmen van *Campylobacter*-positieve vleeskuikens. Daarnaast is de correlatie tussen de kolonisatieniveaus in de krop en de blinde darmen bepaald om te bepalen of het niveau in het enige deel voorspelbaar is voor het niveau in het andere. Zowel in de krop als in de blinde darmen heeft supplementatie van vluchtige vrije vetzuren niet geleid tot een verlaging van kolonisatieniveaus, en werd er geen correlatie tussen beide delen van het spijsverteringsstelsel waargenomen.

Kolonisatie-eigenschappen van *Campylobacter* worden normaliter bestudeerd in kippen. Een kolonisatiemodel in kippenembryos zou een goed alternatief kunnen zijn, aangezien het gelijkenis vertoont met het micromilieu in de darmen van het pasgeboren kuiken en daarmee de onderzoeker in staat stelt om zowel de potentie tot kolonisatie als de interactie tussen bacterie en gastheer te bestuderen in afwezigheid van een darmflora. Om de bruikbaarheid van het embryo kolonisatiemodel te bestuderen werden in **hoofdstuk 6** de kolonisatiekarakteristieken bestudeerd in de darmen van 16 dagen bebroede kippeneieren. In het duodenum (twaalfvingerige darm) en de blinde darm werd een positieve correlatie aangetoond tussen de inoculatiedosis en de kolonisatieniveaus op 1 dag na inoculatie. Na twee dagen werden de maximale kolonisatieniveaus bereikt, welke constant bleven tot dag 3 na inoculatie. De waargenomen kolonisatieniveaus suggereren dat het embryomodel groeiomstandigheden biedt die

een gestage groei van *Campylobacter* mogelijk maakt, waarbij in de cloaca kolonisatieniveaus bereikt worden die overeenkomen met de niveaus in het levende kuiken. Daarnaast werd aangetoond dat de analytische sensitiviteit, gedefinieerd als de kans op kolonisatie na inoculatie van 1 cfu *Campylobacter*, 0.35 en 0.7 bedroeg in de twee uitgevoerde experimenten. Beide waarden zijn vele malen hoger dan de gemiddelde waarde vastgesteld in levende kippen in **hoofdstuk 4**. Concluderend kan gesteld worden dat het embryomodel een gevoelige methode is voor het aantonen van *Campylobacter*.

SLOTOPMERKINGEN

Verscheidene methoden en modellen zijn gebruikt, geëvalueerd en verbeterd in dit proefschrift. Afhankelijk van het doel van toekomstige studies naar de pathogenese van *Campylobacter* of het doel van interventiemaatregelen zouden de beschreven modellen en methoden bruikbaar kunnen zijn. Het transmissiemodel (**hoofdstuk 2 en 3**) is geschikt voor de bestudering van factoren die transmissie beïnvloeden en om introductie routes te identificeren middels het schatten van t_0. Het dosis response model (**hoofdstuk 4**) en het kolonisatieniveau model (**hoofdstuk 5**) zijn geschikt om interventiemaatregelen te evalueren die respectievelijk de gevoeligheid en de kolonisatieniveaus verlagen. Het embryomodel (**hoofdstuk 6**) zou gebruikt kunnen worden als een meer gevoelige test voor het aantonen van *Campylobacter* of voor de bestudering van de pathogenese van *Campylobacter*.

Het is onwaarschijnlijk dat pluimveegerelateerde *Campylobacter* infecties in mensen voorkomen kunnen worden middels enkele preventieve maatregelen in de vleeskuikenhouderij. Verbetering van hygiënemaatregelen op het bedrijf zullen zeker bijdragen aan een beperking van de blootstelling van vleeskuikens. Om het aantal *Campylobacter*-positieve koppels verder te reduceren zijn aanvullende maatregelen, zoals voersupplementatie met MCFA, waarschijnlijk nodig. Aangezien het nu mogelijk is om het moment van introductie nauwkeurig te schatten, kan de analyse van risicofactoren en daarmee de evaluatie van interventie maatregelen voortaan gerichter plaatsvinden. Door het combineren van eerder beschreven preventieve maatregelen, nieuwe maatregelen omschreven in dit proefschrift en toekomstige maatregelen gericht op risicofactoren die geïdentificeerd kunnen worden door schatting van t_0 zou het mogelijk moeten zijn om reductie van pluimveegerelateerde humane blootstelling te realiseren.

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Twan

Curriculum Vitae

Twan van Gerwe was born in Veghel, the Netherlands, on 30 January 1974. He grew up in Erp and graduated from Atheneum B (Zwijsen College Veghel) in 1992. From 1992 till 1993 he studied 'Veehouderij' at the Agrarische Hogeschool in ´s-Hertogenbosch. In 1993 he started the veterinary study at Universiteit Utrecht, where he graduated in 2000. He started his veterinary career at Veterinair Centrum Someren where he was a poultry veterinarian till March 2004. In 2002 he started the MSc course 'Veterinary Epidemiology and Economics' at Universiteit Utrecht (UU), which he finished successfully in 2004. April 2004 - Augustus 2009 he was employed as junior lecturer/PhD student/veterinarian at the Department of Farm Animal health (Faculty of Veterinary Medicine - UU). During this period he conducted the PhD research project described in this thesis.

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