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Acidification of drinking water inhibits indirect transmission, but not direct transmission of *Campylobacter* between broilers

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ABSTRACT

In this study the effect of acidification of the drinking water of broiler chickens on both direct and indirect transmission of *Campylobacter* was evaluated. In the direct transmission experiment both susceptible and inoculated animals were housed together. In the indirect transmission experiment the susceptible animals were spatially separated from the inoculated animals and no direct animal to animal contact was possible. The transmission parameter β was estimated for the groups supplied with acidified drinking water and for the control groups. The results showed that acidification of the drinking water had no effect on direct transmission ($\beta = 3.7\,\text{day}^{-1}$ for both control and treatment). Indirect transmission however was influenced by acidification of the drinking water. A significant decrease in transmission was observed ($p < 0.05$), with control vs. treatment point estimates being $\beta = 0.075\,\text{day}^{-1}$ vs. $\beta = 0.011\,\text{day}^{-1}$.

Apart from providing quantitative estimations of both direct and indirect transmission of *Campylobacter* in broilers, this study also demonstrates the use of an experimental setup for indirect transmission of *Campylobacter* between broilers to assess the efficacy of candidate measures to reduce transmission.

1. Introduction

Campylobacter causes a substantial number of cases of human gastroenteritis worldwide (Allos, 2001; Tauxe, 2002). The handling and consumption of contaminated poultry products are major risk factors for campylobacteriosis (Saleha et al., 1998). Implementation of measures to control *Campylobacter* in the poultry production chain may reduce the exposure of humans to *Campylobacter*. Such measures can be applied either at the slaughterhouse level, i.e. improving the slaughterhouse hygiene, or they can be applied at primary production level, i.e. on farm hygiene and biosecurity measures, to reduce the incidence of *Campylobacter* colonized flocks. A reduction in the number of colonized poultry flocks will decrease the risk for consumers considerably (EFSA, 2010). One way of reducing the number of colonized poultry flocks is by altering the susceptibility of the host; i.e. the chance of successful colonization after exposure (Byrd et al., 2001). In broiler chickens, fermented liquid feed (FLF) has been shown to reduce the susceptibility to *Campylobacter* and *Salmonella* (Heres et al., 2003a,b; Savvidou et al., 2009). In FLF, lactobacilli are present whose main metabolic products are lactic acid and acetic acid (Giraffa et al., 2010). The effects of FLF are attributed to the high level of organic acids and

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the low pH of this feed. Following this line of reasoning, acidified drinking water may be expected to have a similar effect on the susceptibility of broilers to *Campylobacter* as FLF.

The aims of this study were (1) to investigate the effect of acidification of the drinking water on both the direct and indirect transmission of *Campylobacter* between broilers and (2) to explore the use of an experimental system of indirect transmission of *Campylobacter* between broilers for assessing the effect of candidate measures against transmission in a controlled setting. With indirect transmission we mean transmission that occurs in a situation where there is no possibility for contact between susceptible and infectious animals, i.e. they are spatially separated.

2. Materials and methods

2.1. Experimental design

2.1.1. Direct transmission experiment

The direct transmission experiment consisted of one control group and one treatment group and was carried out in duplicate, resulting in four groups in total. Each group consisted of 23 animals. Throughout the experiment the groups were housed in separate stables. From day 0 (day of hatching) until day 12 all animals in a group were housed together. The two control groups received tap water whereas the treatment groups continuously received acidified drinking water. A commercially available acid (Forticoat®, Selko BV) was diluted until a final pH of 4 (approximately 2 ml acid on 1 L water). Active ingredients of the commercially available acid are: sorbic acid, formic acid, acetic acid, lactic acid, propionic acid, ammonium formate, L-ascorbic acid, citric acid, mono- and diglycerides of edible fatty acids and 1,2-propanediol. At day 12, ten animals per group were randomly selected from each group, inoculated with *Campylobacter* by gavage (see Section 2.4) and housed separately. On day 16 the inoculated animals were placed back with the rest of their group. Colonization was monitored by taking cloacal swabs on a daily basis from day 14 onwards. The swabs were processed within 2 h for the analysis of the presence of *Campylobacter*. If an animal was found positive on 5 consecutive days, swabs were taken only once a week. The experiment was ended 20 days post inoculation. At that day all chickens were euthanized and caecal contents were qualitatively analysed for the presence of *Campylobacter*.

2.1.2. Indirect transmission experiment

The indirect transmission experiment consisted of one control group and one treatment group and was carried out in duplicate. Each group consisted of 9 animals. The two control groups received tap water, the treatment groups received acidified drinking water (Forticoat®, Selko BV, pH 4). From day 0 (day of hatching) until day 4 all animals in a group were housed together. On day 4, animals were housed individually according to the housing plan depicted in Fig. 1. This setup was chosen to equalize the infection pressure experienced by each susceptible bird as much as possible. Twelve days after hatching 5 animals from each group were orally inoculated with 1 ml of *Campylobacter* (see Section 2.4). To monitor colonization, from day 12 onwards, swabs were taken on a daily basis from all animals, both inoculated and susceptible. If an animal was found positive for *Campylobacter* on 5 consecutive days, swabs from that animal were taken on a weekly basis. The experiment was ended 21 days post inoculation. All animals were euthanized and the caeca were removed and qualitatively analysed for the presence of *Campylobacter*.

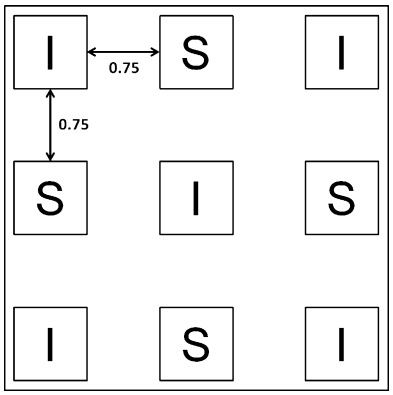

Fig. 1. Schematic overview of the housing of the animals during the indirect transmission experiment. S: susceptible animal; I: infectious animal. Distances are given in metres.

2.2. Housing

Animals were housed in wire cages placed directly on the floor. Wood shavings were provided as bedding material; feed was supplied ad lib.; drinking water was supplied via an open water drinking system. No flow of water was possible between infectious and susceptible animals. Drinking water was refreshed on a regular basis. Before the start of the experiment all stables used in the experiment were cleaned and disinfected and samples were taken from different areas inside the stable, to check for the absence of *Campylobacter*.

2.3. Animals

Eggs from commercial broilers (type Ross 308) were incubated in an in-house facility. Day of hatching is day 0 in the experiment. On day 1 and day 8 cloacal swabs were taken from all animals to check for the absence of *Campylobacter*. These samples were incubated in mCCD (modified cefoperazone charcoal deoxycholate) broth (Nutrient Broth no. 2, Oxoid CM0067 with *Campylobacter* selective supplement (Oxoid SR0204E) and *Campylobacter* growth supplement (Oxoid SR0232E)) for 24 h and plated on mCCDA (modified cefoperazone charcoal deoxycholate agar) and incubated again to check suspected *Campylobacter* colonies after 24 and 48 h (see Section 2.5 for complete procedure). All animals were uniquely tagged so they could be tracked throughout the experiment.

All animal experiments were in compliance with national and institutional regulations and as such approved by the institute's ethical committee.

Table 1
Results for the direct transmission experiment. Total number of observed colonized broilers, corresponding day number of colonized broilers per stable and per treatment estimate of transmission parameter β are shown. p.i.: post inoculation.

Stable	Type of drinking water	Observed colonized broilers (Total animals)	Day numbers of observed colonized broilers (p.i.)	β (95% C.I.)
1	Normal tap water	13 (13)	1	n.a.[a] (2.5–∞)
2	Normal tap water	13 (13)	1	
3	Acidified tap water	13 (13)	1, 2	3.7 (2.0–6.8)
4	Acidified tap water	13 (13)	1	

[a] All animals were found positive for *Campylobacter* on the first day of sampling, therefore, the point estimate for the transmission parameter is unidentifiable from the available data.

2.4. Inoculum

The *Campylobacter* strain used in this experiment was *Campylobacter jejuni* strain C356, originally isolated from broilers (Jacobs-Reitsma et al., 1994). The strains were freshly cultured in hearth infusion broth (microaerobically, 37 °C, overnight) and diluted in buffered peptone water to obtain the intended inoculation dose (10^5 CFU/ml).

2.5. Sampling

Samples were collected using sterile swabs. Swabs were directly plated on mCCDA, these plates where incubated microaerobically at 41.5 °C for 48 h and examined for the presence of *Campylobacter*. After plating the swabs were placed in an enrichment medium (CCD broth) and incubated microaerobically at 41.5 °C for 24 h. After incubation 10 μl was plated on mCCDA and incubated microaerobically at 41.5 °C for 48 h and examined for the presence of *Campylobacter*. Sensitivity and specificity for testing cloaca swabs were estimated as both being very close to 1 (personal communication R. van der Hulst, CVI, Lelystad).

2.6. Quantification of transmission

A stochastic susceptible-infectious (SI) type model was used to describe the transmission between inoculated animals (seeders) and susceptible animals (contact animals).

In the SI-model individuals in a population of size N are either susceptible (S) or infected (I). Susceptible individuals get infected with rate $\beta SI/N$, where β is the transmission parameter. Substituting S by N-I and given a sufficiently small time interval Δt, it is possible to formulate separate differential equations for the probability of finding the population in every possible state (Keeling and Rohani, 2008; Allen, 2010).

These master equations (or state probabilities) can be written in matrix form: $d\vec{P}/dt = Q \times \vec{P}$, with generator matrix:

$$Q = \begin{pmatrix} -\beta(N-1)/N & 0 & \cdots & 0 & 0 \\ \beta(N-1)/N & -2\beta(N-2)/N & \cdots & 0 & 0 \\ 0 & 2\beta(N-2)/N & \cdots & 0 & 0 \\ \vdots & \vdots & \cdots & \vdots & \vdots \\ 0 & 0 & \cdots & -\beta(N-1)/N & 0 \\ 0 & 0 & \cdots & \beta(N-1)/N & 0 \end{pmatrix}$$

and their solution is: $\vec{P}(t) = e^{Qt} \cdot \vec{P}_0$. A further explanation and an implementation of this method are given in the appendix (electronic supplement).

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Using this solution, the probability of the state observed at each sampling moment, conditional on the state observed at the previous sampling moment, can be calculated in an exact manner provided that the sensitivity and specificity of the cloaca swabbing are 1. Thus β can be estimated using a maximum likelihood approach and exact confidence bounds can be obtained by summing the probabilities of all scenarios that are as extreme as or more extreme than the observed scenario. All calculations were implemented and performed in Mathematica 7.0 (Wolfram Research, 2010).

3. Results

The results of the direct transmission experiment are given in Table 1. For both control and treatment, after the inoculated (and infectious) animals were placed back with the susceptible animals, all susceptible animals became colonized with *Campylobacter*. The colonization of the

Table 2
Results for the indirect transmission experiment. Total number of observed colonized broilers, corresponding day number of colonized broilers per stable and per treatment estimate of transmission parameter β are shown. p.i.: post inoculation.

Stable	Type of drinking water	Observed colonized broilers (Total animals)	Day numbers of observed colonized broilers (p.i.)	β (95% C.I.)
1	Normal tap water	3 (4)	12, 12, 14	0.075 (0.027–0.16)
2	Normal tap water	2 (3)	15, 18	
3	Acidified tap water	1 (4)	20	0.011 (0.0006–0.047)
4	Acidified tap water	0 (4)	–	

contact animals occurred rapidly: within 1 day for all but one (an animal in one of the treatment groups which became colonized on day 2). The estimation of the transmission parameter β with the maximum likelihood procedure yielded a value of 3.7 (95% C.I.: 2.0–6.8) per day. No significant differences in the transmission parameter were found between control and treatment groups (Wald-test, $p = 0.9$) and the data of the two groups were pooled in subsequent analyses.

For the indirect transmission experiment the number of transmission events (colonization) per stable is given in Table 2. These results show that indeed indirect transmission of *Campylobacter* between spatially separated broilers occurred. Furthermore fewer colonized animals in the treatment stables compared to the control stables were found and, when tested with a one-sided Fisher exact test, this difference was found to be significant (Fisher exact, $p = 0.035$). A one-sided test was used here because we did expect less animals to be colonized in the treatment stables. One animal died in stable 2; this animal was excluded from the analysis.

The estimates obtained for the transmission parameter β are shown in the last column of Table 2. For the control groups we found an estimate for β of 0.099 (95% C.I.: 0.035–0.21) per day. The estimate found for the treatment groups was 0.011 (95% C.I.: 0.0006–0.049) per day. Based on a Wald-test the difference between the control and treatment groups was significant ($p < 0.05$), indicating that acidification of the drinking water reduced the transmission parameter.

4. Discussion

This study was carried out to determine whether acidification of the drinking water has an influence on the transmission of *Campylobacter*. Both direct and indirect transmission (transmission between spatially separated broilers) was investigated. As we used a novel experimental setup with spatially separated broilers to study indirect transmission, this study also served to explore its use as a system to test possible measures to reduce indirect transmission.

Our results showed that acidification of the drinking water had no effect on the direct transmission of *Campylobacter* between broilers; however, there was a significant reduction in transmission between spatially separated broilers (i.e. indirect transmission) when the drinking water was acidified.

Three hypotheses may explain the effect of acidification of the drinking water on indirect transmission. First, the host animal might be less susceptible for *Campylobacter* colonization due to acidification of the drinking water. The basis of this is that gizzard and stomach of the chickens become more acidic when the animals receive acidified drinking water. This might reduce the number of bacteria that reach the lower parts of the gastrointestinal tract. Bjerrum et al. demonstrated a similar effect in broilers fed with whole wheat (Bjerrum et al., 2005), which has an acidifying effect in the gizzard. They showed that broilers fed with whole wheat had significantly reduced numbers of *Salmonella typhimurium* in the gizzard and ileum. However no difference with respect to the number of *Salmonella* was found in the caeca and rectum. As a second hypothesis there is the possibility that due to the acidification of the drinking water, the actual number of bacteria per gram faeces shedded by the inoculated animals is less compared to a control situation, eventually leading to a decreased probability of colonization of the susceptible animals. A third hypothesis involves the environment the bacteria pass through on their way from the shedding animal to the receiving susceptible animal. Once shedded via the faeces, the bacteria enter a more hostile environment, due to the acidification, and the dying off in the environment increases, thereby decreasing the probability of colonization. With the current experiment we cannot distinguish between these three hypotheses and further research is needed to identify the correct mechanism(s). As the three hypotheses are not mutually exclusive also a combination of two or three is possible as an explanation for the effect observed.

Also the exact route of indirect transmission remains unknown. For example, dust, litter or animal care-takers are just some possible routes of transmission. More research about the exact routes of transmission is needed to determine and classify routes of indirect transmission. Indeed for some infectious diseases this has been attempted (Stegeman et al., 1999; Stärk, 2000; Ribbens et al., 2007), however for many diseases the actual routes and the contribution of the different routes remain unclear.

As the indirect transmission experiment carried out in this study mimics a between-flock transmission situation, the findings may indicate that acidification of the drinking water might have a reducing effect on between-flock transmission. In a modelling analysis of interventions, Katsma et al. showed that the most effective method to reduce the *Campylobacter* prevalence is to reduce the between-flock transmission (Katsma et al., 2007). This underlines the potential effect of acidification of the drinking water as a possible control measure. It should be noted however that our direct transmission experiment showed that the direct transmission of *Campylobacter* is not affected by acidification of the drinking water. Therefore once *Campylobacter* is introduced into a flock it will still spread fast within this flock ($\beta = 3.7\ \text{day}^{-1}$), although some care must be taken when extrapolating from an experimental setup as in this study to a full commercial flock. It should be noted that the sample size in this study is relatively small, resulting in the parameter estimation being sensitive to small differences in number of infected animals. To get more robust parameter estimates more replications of this study should be performed.

The main conclusions of these experiments are that direct transmission (within-flock transmission) of *Campylobacter* between broilers is not altered by acidification of the drinking water; however, acidification of the drinking water has a decreasing effect on the indirect transmission of *Campylobacter* between broilers. Whether this effect is large enough to contribute meaningfully to the transmission of *Campylobacter* between flocks needs to be studied under field conditions. The results of these experiments also show that our experimental setup for indirect transmission of *Campylobacter* between broilers is a promising

approach for evaluating candidate measures for the reduction of transmission.

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