



## Phage therapy reduces *Campylobacter jejuni* colonization in broilers

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### Abstract

The effect of phage therapy in the control of *Campylobacter jejuni* colonization in young broilers, either as a preventive or a therapeutic measure, was tested. A prevention group was infected with *C. jejuni* at day 4 of a 10-day phage treatment. A therapeutic group was phage treated for 6 days, starting 5 days after *C. jejuni* colonization of the broilers had been established. Treatment was monitored by enumerating *Campylobacter* colony forming units (CFU) and phage plaque forming units (PFU) from caecal content. Counts were compared with control birds not receiving phage therapy. A clear 3 log decline in *C. jejuni* counts was initially observed in the therapeutic group, however, after 5 days bacterial counts stabilized at a level 1 log lower than that of the control group. Colonization of *C. jejuni* in the prevention group was delayed by the treatment and after an initial 2 log reduction, colonization stabilized within a week at levels comparable to the therapeutic group. The CFU and PFU counts displayed opposing highs and lows over time, indicative of alternating shifts in amplification of bacteria and phages. There were no adverse health effects from the phage treatment. Two different phages were combined as therapeutic treatment of *Campylobacter* positive chickens challenged at the age approaching broiler harvest. This again resulted in a significant decrease in *Campylobacter* colonization. We conclude that phage treatment is a promising alternative for reducing *C. jejuni* colonization in broilers.

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### 1. Introduction

*Campylobacter jejuni* is an important human pathogen and is the most common cause of bacterial

gastroenteritis worldwide (Lindqvist et al., 2001; Adak et al., 2002; Samuel et al., 2004). Poultry flocks are frequently colonized with *C. jejuni* without apparent symptoms (Shane, 2000) and risk assessment analyses have identified handling and consumption of poultry meat as one of the most important sources of human campylobacteriosis (Evans et al., 2003; Potter et al., 2003; Friedman et al., 2004).

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Attempts to prevent *Campylobacter* colonization of chickens by biosecurity measures have proven extremely difficult. At best, colonization can be delayed, but not prevented (Gibbens et al., 2001; Hartnett et al., 2001). The ubiquitous presence of *C. jejuni* in the environment and in warm-blooded animals including pets, rodents, and wild birds is probably responsible for this lack of success (Newell and Fearnley, 2003). Several other prevention strategies have been proposed. The effect of experimental vaccination of birds was assessed, but the organisms are not cleared during the life span of broilers (Khoury and Meinersmann, 1995; Rice et al., 1997), and vaccination is hampered by the large serotypic diversity of *C. jejuni* strains. Probiotic treatment with lactic acid bacteria and competitive exclusion with beneficial microflora was only partially effective (Mead, 2000; Chen and Stern, 2001). Treatment of drinking water with acids or acidic feed additives has a limited beneficial effect (Heres et al., 2004). When a *Campylobacter* positive flock enters the slaughter house (cross-) contamination of meat is highly likely (Rivoal et al., 1999; Newell et al., 2001). Thus, there is an urgent need for control measures that can be used in the field and that are acceptable to consumers.

The use of bacteriophages to combat bacterial infections has regained general interest now that resistance to antibiotics has become a serious problem (Barrow and Soothill, 1997; Alisky et al., 1998; Merrill et al., 2003). Bacteriophages are highly specific for bacterial species, and multiply at the expense of the cell, eventually reducing the number of viable bacterial cells (Carlton, 1999; Sulakvelidze and Morris, 2001). Due to their host specificity, effects on other microbial populations are minimal. The use of bacteriophages has been investigated to reduce *Campylobacter* load on chicken skin after slaughter (Goode et al., 2003; Atterbury et al., 2003a). However, activity of bacteriophages is maximal at the optimal growth temperature of their host (Hudson et al., 2005), which is 40 °C in case of *Campylobacter*.

Here, we investigated the application of phage therapy in live broilers in two possible applications: therapeutic use and preventive use. The results indicate that phage therapy decreases colonization levels by several orders of magnitude and thus

demonstrates its potential in the control of human campylobacteriosis.

## 2. Materials and methods

### 2.1. Birds, housing and experimental setup

One day old Ross broiler chickens were housed in groups in separate isolators. All birds within a group received identical treatment. Feed and water was supplied ad libitum. The experiments were conducted according to the animal welfare regulations, and approved by an independent ethical committee. Two sets of experiments were conducted with treatment of (set I) 10-day old chickens to be monitored through to day 42, which is the usual age for broilers at slaughter, and (set II) 32-day old chickens, to be monitored for 26 days, respectively.

#### 2.1.1. Experiment I

Set I comprised of four groups of birds that were all checked for the absence of *Campylobacter* and *Salmonella* by cloacal swabbing at days 2 and 9. All 52 birds of group A (preventive treatment) received phage administration by oral gavage starting 4 days prior to as well as during *C. jejuni* oral challenge colonization. An overview of the treatment is given in Table 1. Each day from day 7 to 16, group A chickens received an oral phage dose (phage strain 71) varying from  $4 \times 10^9$  to  $2 \times 10^{10}$  plaque forming units (PFU) and at day 10 an oral *C. jejuni* challenge of  $1 \times 10^5$  CFU. Group B, 48 birds for the therapeutic treatment, received an oral dose of  $1 \times 10^5$  CFU *C. jejuni* on day 10, followed by inoculation with phage strain 71 for 6 successive days (varying from  $9 \times 10^9$  to  $1 \times 10^{10}$  PFU) for days 15–20, starting 5 days after the *Campylobacter* administration (Table 1). All 35 animals of group C (phage-only control group) received identical doses of phage 71, and at the same days as group A. These animals were not challenged with *C. jejuni*. All 64 birds of group D (*C. jejuni* only control) were challenged with *C. jejuni* at day 10 and did not receive phage treatment. The scheme of inoculation of all 4 groups with time and doses is given in Table 1.

Table 1  
Scheme of infection and dose of experimental set I

Day <sup>a</sup>	Group A		Group B		Group C	Group D
	Phage dose <sup>b</sup>	<i>C. jejuni</i> dose <sup>c</sup>	Phage dose <sup>b</sup>	<i>C. jejuni</i> dose <sup>c</sup>	Phage dose <sup>b</sup>	<i>C. jejuni</i> dose <sup>c</sup>
7	$5 \times 10^9$				$5 \times 10^9$	
8	$4 \times 10^9$				$4 \times 10^9$	
9	$9 \times 10^9$				$9 \times 10^9$	
10	$1 \times 10^{10}$	$1 \times 10^5$		$1 \times 10^5$	$1 \times 10^{10}$	$1 \times 10^5$
11	$1 \times 10^{10}$				$1 \times 10^{10}$	
12	$1 \times 10^{10}$				$1 \times 10^{10}$	
13	$1 \times 10^{10}$				$1 \times 10^{10}$	
14	$1 \times 10^{10}$				$1 \times 10^{10}$	
15	$1 \times 10^{10}$		$1 \times 10^{10}$		$1 \times 10^{10}$	
16	$2 \times 10^{10}$		$9 \times 10^9$		$2 \times 10^{10}$	
17			$9 \times 10^9$			
18			$9 \times 10^9$			
19			$9 \times 10^9$			
20			$9 \times 10^9$			

<sup>a</sup> Day of hatching is given as day 0.

<sup>b</sup> Dose of phage 71 given as PFU.

<sup>c</sup> Dose given as CFU.

### 2.1.2. Experiment II

For experimental set II, birds were kept in isolators from the day of hatching and were randomly checked for absence of *Campylobacter* and *Salmonella* at days 15 and 26 by cloacal swabs. At day 31 all birds were tested again for *Salmonella* and *Campylobacter*, and divided in three groups. Group E, comprising 36 birds kept in four isolators with 9 birds each, received *C. jejuni* challenge (at day 32) followed by phage administration 7 days later. Two different phage types (phage strains 69 and 71) were given orally to each bird of group E and to the birds of the control group G. An overview of the treatments is summarized in Table 2. Group F was a *C. jejuni* only control group of the same size as

group E. Group G was a phage-only control group of 10 animals in one isolator.

### 2.2. *C. jejuni* strain and growth conditions and inoculation of animals

The *C. jejuni* challenge strain C356 used in this study was originally isolated from a commercial broiler in The Netherlands in 1991 (Jacobs-Reitsma et al., 1995). The strain had been stored at  $-80^\circ\text{C}$  and in previous experiments was shown to have retained good colonizing properties (results not shown). Bacteria were grown micro-aerobically (6%  $\text{O}_2$ , 7%  $\text{CO}_2$ , 7%  $\text{H}_2$ , 80%  $\text{N}_2$ , Anoxomat, Mart Microbiology B.V., The Netherlands) at  $42^\circ\text{C}$  on

Table 2  
Scheme of infection and administered dose of experimental set II

Day <sup>a</sup>	Group E			Group F	Group G	
	<i>C. jejuni</i> dose <sup>b</sup>	Phage 71 dose <sup>c</sup>	Phage 69 dose <sup>c</sup>	<i>C. jejuni</i> dose <sup>b</sup>	Phage 71 dose <sup>c</sup>	Phage 69 dose <sup>c</sup>
32	$1 \times 10^5$			$1 \times 10^5$		
39		$4 \times 10^{11}$	$3 \times 10^{10}$		$4 \times 10^{11}$	$3 \times 10^{10}$
40		$2 \times 10^{10}$	$5 \times 10^9$		$2 \times 10^{10}$	$5 \times 10^9$
41		$5 \times 10^{10}$	$2 \times 10^{10}$		$5 \times 10^{10}$	$2 \times 10^{10}$
42		$4 \times 10^{10}$	$2 \times 10^{10}$		$4 \times 10^{10}$	$2 \times 10^{10}$

<sup>a</sup> Day of hatching is given as day 0.

<sup>b</sup> Dose given as CFU.

<sup>c</sup> Dose given as PFU.

heart infusion agar supplemented with 5% sheep blood (HIS agar). Bacteria were harvested after 48 h of culture in phosphate buffered saline (PBS), washed once and, based on OD<sub>600</sub>, diluted to approximately  $5 \times 10^5$  CFU/ml. Chickens were dosed individually by gavage with 250  $\mu$ l bacterial suspension orally. The exact dose was confirmed by serial dilutions cultured on HIS agar and is given in Tables 1 and 2.

### 2.3. Phage type and growth conditions

Bacteriophage 69 (NCTC 12669) and bacteriophage 71 (NCTC 12671) both belong to the *Myoviridae* and are lytic for *C. jejuni* (Sails et al., 1998). The phages were obtained from the National Collection of Type Cultures in the UK. Phages used for administration to chickens were cultured on *C. jejuni* C356 in Complex media broth (Leach et al., 1997) at 42 °C for 20 h to a titre of approximately  $1 \times 10^{10}$  to  $1 \times 10^{11}$  PFU/ml. The exact number of plaque forming units (PFU) was determined by titration as given below. Animals were individually administered with 250  $\mu$ l of phage suspension orally by gavage. In experimental set I, phage 71 was used alone. In experimental set II, phage 69 was dosed 3 h after phage 71 (which was administered at a higher dose on the first day) and then both phage strains were given together on the consecutive dosing days.

### 2.4. Phage counts by phage-spot-agar and PFU determination

Prior to accurate titer determination, the phage titer was estimated on phage-spot-agar plates. These plates were prepared by mixing 0.3 ml of an overnight *C. jejuni* C356 culture with top agar at 50 °C (0.7% agar in BHI-broth supplemented with 10 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.01% sodium bisulfite). Five ml of the mixture were poured into an empty petri dish and 10  $\mu$ l of a 10 $\times$  dilutions series (in PBS containing 0.1% Tween80) were spotted on the lawn after the agar solidified. The dilutions were stored overnight at 4 °C.

Based on the spot plate results, the dilution which gave approximately 100 plaques was used the next day for exact PFU determination in triplicate. Ten  $\mu$ l of that dilution was mixed with 200  $\mu$ l overnight *C. jejuni*-culture (diluted to OD<sub>600</sub> = 0.1), incubated at

room temperature for 15 min, mixed with 5 ml top agar (50 °C) and poured into petri dishes. The plates were incubated for 18–28 h at 42 °C under micro-aerobic conditions. Phage plaques were counted and the triplicates were averaged.

### 2.5. Necropsies, harvesting and processing of caecal contents

#### 2.5.1. Experiment I

At each day of necropsy, for each group 4 chickens were taken from the isolators and euthanised with an intracranial injection of T61<sup>®</sup> (Intervet, Boxmeer, The Netherlands).

Necropsies of animals from group A were performed at days: 11, 12, 13, 14, 15, 16, 19, 22, 25, 29, 32, 36, and 39. Animals from group B were killed at days 15, 16, 17, 18, 19, 20, 22, 25, 29, 32, 36, and 39. Animals from group C were killed at days 9, 11, 12, 13, 16, 19, 22, 25, 29, 32, 36, and 39. Animals from group D were killed at each of the days that either animals from group A or group B were killed, as they served as control animals for *Campylobacter* counting.

#### 2.5.2. Experiment II

Necropsies of animals of group E and group F were performed at days 38, 39, 40, 41, 42, 43, 44, 46, 49, and 58. At each day of necropsy, for each group 4 chickens were randomly chosen from the isolators and euthanised as above. For Group G, necropsies on two animals were performed at days 38, 39, 43, 46, and 49.

### 2.6. Caecal contents and sample processing

The caeca of the animals were collected and 1 g of contents was diluted in PBS resulting in a 10<sup>-1</sup> dilution. For bacterial CFU counts, 50  $\mu$ l was mixed in 96-well plates with 50  $\mu$ l of anti-phage antibody (polyclonal antisera obtained from a rabbit immunized with phage 71 with reactivity to both phage 71 and 69 (data not shown)). Anti-phage antibody was added to the 10<sup>-2</sup> dilution too. The mixture was incubated for 20 min at room temperature to inactivate phages present before further serial dilutions (10<sup>-3</sup> to 10<sup>-7</sup>) in PBS were made, of which 100  $\mu$ l was plated for CFU determination of *Campylobacter* on CCDA plates (Campylobacter Blood Free Selective Medium,

CM739) supplemented with cefoperazone (32 mg/l) and nystatin amphotericin B (10 mg/l) (Oxoid, CCDA Selective Supplement SR 155). The plates were incubated at 42 °C for 18–48 h under microaerobic conditions until colonies were large enough to be counted. Serial dilution plates containing 30–300 colonies were used for CFU determination. At regular intervals typical colonies were examined microscopically (Gram-staining and motility) for *Campylobacter* confirmation. At each of the indicated days of necropsy, samples from four individual animals were analysed for CFU. For phage PFU counts, 40 µl/ml chloroform was added to the 10<sup>-1</sup> caecal contents dilution, and the vortexed mixture was incubated for 20 min at room temperature, centrifuged at 8000 rpm for 10 min, and filtered through a 0.45 µm filter. From this filtered dilution a serial dilution was made into PBS and 10 µl was used from each dilution for phage-spot-agar plates as described above. Two to four animals were analysed for PFU at necropsy days.

### 3. Results

#### 3.1. Comparing therapeutic and preventive phage treatment in young chickens

Bacteriophage 71 was chosen since it displayed activity cross a wide range of *C. jejuni* strains. In a preliminary test comparing the sensitivity of 27 poultry-derived *C. jejuni* strains to 14 different phages, phage 71 displayed the widest activity (results not shown).

Ten-day old chicks were administered with *C. jejuni* C356, historically a good colonizing strain, before (group B) and after (group A) treatment with phage 71, to assess the therapeutic and preventive effect, respectively. For group A, phages were administered as one dose on each of 10 consecutive days. At regular intervals, the number of *C. jejuni* CFU and phage PFU in caecal contents were determined from individual chicks. As shown in Fig. 1, phage treatment prior to bacterial challenge (group A) could not prevent but did delay bacterial colonization. Nevertheless, phages established themselves in the caeca of the birds of group A, as they could be isolated for up to 24 days after receiving the last phage dose. During this time, numbers of *C. jejuni* detected in the

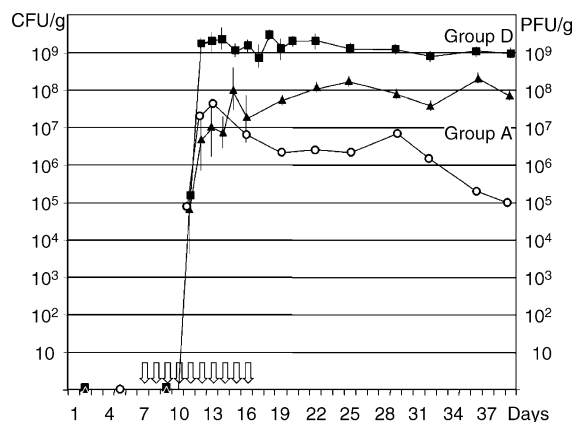


Fig. 1. *C. jejuni* strain C356 colonization (CFU per gram faeces) and the presence of phage 71 (PFU per gram faeces) in young chickens receiving 'preventive' treatment with phage 71. The CFU data are indicated as an average of four animals with the S.D. The triangles represent *C. jejuni* colonization in the treated animals of group A (preventive) and the squares the *C. jejuni* colonization in the control animals (group D). The PFU (phage 71) are presented by circles. The days at which chicks were dosed with phage are indicated by arrows. *C. jejuni* was given at day 10.

caecum remained lower than in the control group not receiving phage. The CFU and PFU counts displayed opposing highs and lows over time, indicative of alternating shifts in amplification of bacteria and phages (Fig. 1). Chicks dosed with *C. jejuni* only (group D) showed levels of colonization approximately 10 times higher than the birds receiving phage treatment (Fig. 1).

Chicks receiving phages after *C. jejuni* colonization had been established showed an immediate 3 log reduction in CFU counts (Fig. 2). PFU counts are also shown in the figure. After the birds had received their last dose of phage, bacterial levels increased, and eventually stabilized at a level comparable to group A. The control group C which received phage only showed that phage 71 was not able to maintain itself in the cecum in absence of *C. jejuni* (Fig. 3). Neither birds from this control group nor birds from the treatment groups showed any adverse effect of the phage therapy.

These findings suggested that phage 71 was at best able to reduce *C. jejuni* colonization by 1 log over the time span of 30 days. However, the initial drop in colonization seen in group B indicated that phages can reduce colonization levels at least 3 log for the first few days.

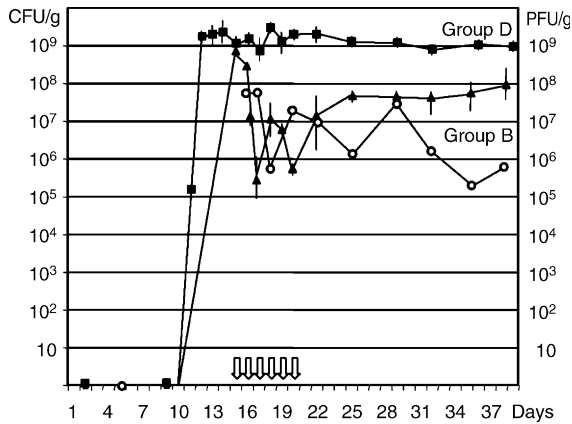


Fig. 2. *C. jejuni* strain C356 colonization (CFU per gram faeces) and the presence of phage 71 (PFU per gram faeces) in young chickens receiving therapeutic treatment with phage 71 after *C. jejuni* colonization had been established. The data are indicated as an average of four animals with the S.D. The triangles represent the treated animals of group B 'therapeutic' and the squares the control animals (group D). The PFU (phage 71) are presented by circles. The days at which chicks were dosed with phage are indicated by arrows. *C. jejuni* was given at day 10.

### 3.2. Therapeutic treatment of chickens nearing harvesting age

The data generated with the first experimental set initiated a second trial, in which older chickens were given phage therapy 10 days before the typical age at which broilers are normally slaughtered. A second phage (strain 69) was added to phage strain 71 in order to determine if a more pronounced decrease in *Campylobacter* colonization could be obtained when two different phage strains were present. Preliminary

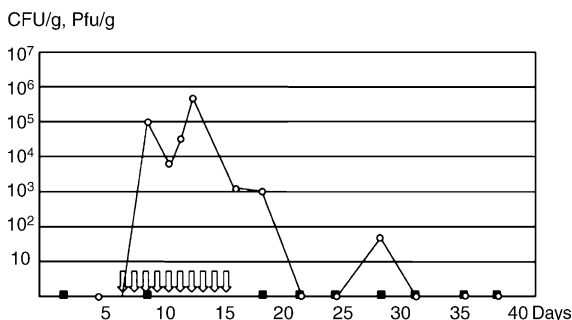


Fig. 3. Phage 71 enumeration (PFU per gram faeces) in young chickens dosed with phage only (group C). In absence of *C. jejuni* (CFU/g, squares), the phage (circles) is not maintained.

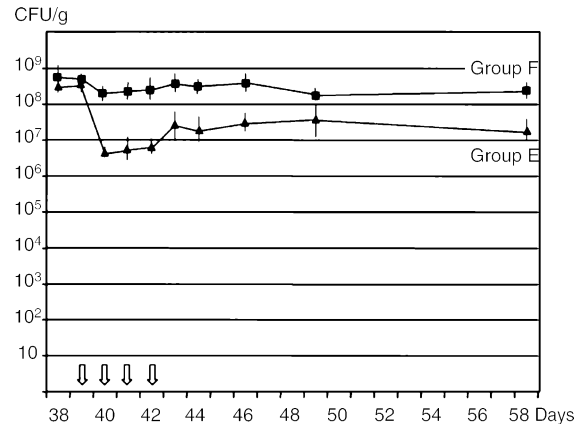


Fig. 4. *C. jejuni* colonization (CFU per gram faeces) in the caecum in elder chickens (dosed at day 32) receiving 'therapeutic' treatment (at the days indicated by arrows) of phage 71 and phage 69 consecutively. Triangles represent phage treated birds (group E) and squares are derived from control birds receiving bacteria only (group F). Each point represent an average of four animals.

tests had shown that phage 69 could kill *C. jejuni* strains refractory to phage 71 (data not shown).

Proven *Campylobacter*-free chickens received a *C. jejuni* challenge at age 32 days, followed by four phage doses of both phage 69 and 71 at days 39, 40, 41, and 42 (Table 2). The birds were sampled for another 2 weeks. The results for the sampled caeca (Fig. 4) show an initial reduction of 1.5 log followed by a slight increase and again an eventual stabilization at colonization levels 1 log lower than that of control birds.

## 4. Discussion

The experimental use of phage therapy to prevent or treat infections with bacterial pathogens has been shown to be successful. For example, diarrhea due to *E. coli* in calves could be prevented or treated with *E. coli*-specific phages (Smith et al., 1987). Similarly, phage treatment was effective in treating experimental *E. coli* septicemia and meningitis in chickens and calves (Barrow et al., 1998). Phage therapy with promising results has been described for treatment of infectious diseases in aquaculture (Nakai and Park, 2002). In experimental bacteremic infections of mice with vancomycin-resistant *Enterococcus faecium*,

phage therapy reduced mortality by 100% (Biswas et al., 2002). Commercial applications for phage treatment of infectious diseases in animals are not yet available, but as veterinary use of antibiotics becomes more controversial due to increased antibiotic resistance, phage therapy may become more generally accepted. To our knowledge this is the first report of phage therapy to decrease the colonization levels of an apparent commensal, *C. jejuni*, in chickens. *Campylobacter* seems to be part of the natural flora of poultry and is harmless to the birds. Although there is a host immune response (antibody titers), this seems to have no significant effect on the colonization levels of *Campylobacter*, at least during the life span of a broiler (Myszewski and Stern, 1990). Even with an improvement of the immune response due to vaccination, the maximal observed effect reported in the literature was a decrease of *Campylobacter* by less than 1 log. Other intervention strategies, such as pre- or probiotics, have been equally disappointing.

Our results show clearly that phage-treatment decreases *C. jejuni* colonization in broiler caeca. This effect is present when phages are used in a preventive way as well as therapeutically, in broilers with an established *C. jejuni* infection. The effect of the phage treatment is most clear in the first days after the treatment (or shortly after the *C. jejuni* infection in prior-phage-treated animals). As the moment of introduction of *Campylobacter* into a flock is very unpredictable, the preventive phage strategy is only of value for experimental conditions. For this reason preventive treatment was not included in the second experiment.

Phage treatment showed no detectable adverse effects in chickens, and phages are able to remain viable while transiting the gastrointestinal tract. No phages were detected in the control group receiving only phage after 7 days (except for one day at which one plaque was observed), indicating that phage clear out of the bird rapidly without a suitable bacterial host. The choice of phage strains used in our experiments excluded concerns about gene transfer by transduction, as the phages used were purely lytic to their host.

After an initial significant reduction of bacterial counts, phage and bacteria eventually reached an equilibrium with bacterial colonization levels 10 times lower than the control group. It could be expected that a resistant subpopulation could develop during

treatment. If this were the case, introducing a second phage in the same treatment protocol would reduce the tendency to develop a resistant population. Against expectations, the addition of a second phage did not significantly assist in the goal of reduction in *C. jejuni* counts. As observed with the use of phage 71 alone, the addition of phage 69 resulted in an ultimate reduction of 1.5 log in CFU counts. However, the experiments are not directly comparable, as dose regime and age of chickens differed significantly between the two experiments. In the second experiment, the conditions more closely resembled that seen with broilers shortly before they are slaughtered. The two phages were tested in vitro for antagonistic effects. When the two phages 71 and 69 were mixed together, the combined titer was that of the two individual phage titers added up (data not shown). Infection of phage 71 with phage 69 in liquid culture produced the same drop at 24 h as did phage 71 alone, indicating that phage 69 did not interfere with phage 71. More testing should be carried out for conclusive evidence of antagonistic effects and to see if phage 69 does help when strains refractory to phage 71 appear (and vice versa). Possibly, the use of more than two phages simultaneously would improve the bacterial reduction at a longer time scale.

An important observation in the first set of experiments is the initial 3 log reduction in bacterial load, directly after introducing the phage. This indicates that careful timing of phage administration one day before slaughter might result in maximum effect by the time the birds are harvested. The optimal phage dose that is most effective for reduction of *Campylobacter* counts needs to be determined. Single dose would be most practical under farm conditions, and supply through drinking water would be preferable. It is important, however, that phage can be completely inactivated in an all-in all-out production system, in order to render the phage effective. In particular, the difficulties with frequent infection of subsequent flocks at the same farm are of concern, as this may indicate that *Campylobacter* resides somewhere at the farm (Connerton et al., 2004). Since the same could happen with the phages introduced during a production cycle and actual farm setting, in the next production cycle, the combined presence of *Campylobacter* with this phage might lead to equilibrium, such that treatment with this same phage just before

slaughter age would not induce the decrease in colonization that is seen when a 'fresh' phage is introduced. Rotating use of different phages would be needed in order to deal with such partial-resistance problem.

Application of phage therapy to production broilers would inevitably release phage-infected *C. jejuni* in the environment and on food. We believe this to be acceptable, since phages have been shown to reside in *Campylobacter* populations present on retail poultry as a result of natural infection (Atterbury et al., 2003b). Quantitative risk assessment modelling suggests that a reduction of 2 log in chickens at the time of slaughter would be sufficient to have a major impact on human incidence of disease (Rosenquist et al., 2003).

In conclusion, the results described here show that it is possible to significantly decrease the number of campylobacter in already-colonized chicken caeca by means of phage therapy. More work will be needed to improve the efficiency of phage therapy, to explore the large scale propagation of phages on pathogenic hosts, and to test the robustness of the method with chicken management systems and with *Campylobacter* strain variation. However, we conclude that the current results merit such investigations.

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