

## Application of Host-Specific Bacteriophages to the Surface of Chicken Skin Leads to a Reduction in Recovery of *Campylobacter jejuni*

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**Retail poultry products are widely purported as the major infection vehicle for human campylobacteriosis. Numerous intervention strategies have sought to reduce *Campylobacter* contamination on broiler carcasses in the abattoir. This study reports the efficacy of bacteriophage in reducing the number of recoverable *Campylobacter jejuni* cells on artificially contaminated chicken skin.**

Since their discovery in the late 1970s, campylobacters have been recognized as a major source of human gastrointestinal disease (11). Poultry, and especially chicken due to its preeminence in the market place, are considered major reservoirs of *Campylobacter* infection (14, 21). This is supported by reports of high numbers of campylobacters colonizing the avian gut (2, 17) and the high incidence of *Campylobacter*-positive poultry and poultry-derived products (15, 16). The rapid spread of *Campylobacter* in broiler house flocks has been previously reported, with one study showing total flock colonization in two days from the initial exposure (12). Although campylobacters are sensitive to desiccation, they are readily recovered from broiler fecal droppings (1). Moreover, this bacterial genus appears to be so well adapted to the avian gut that as few as 100 organisms can colonize (25). These two factors not only facilitate rapid flock colonization but also serve to disseminate the organism inside the broiler house and over the external surface of the birds.

Campylobacters can frequently be isolated from the feathers and skin of broiler chickens (3). One method of reducing broiler carcass contamination involves the use of hyperchlorite in scald water, for carcass washes, and chilled water tanks. Previous studies have shown this treatment to be ineffective in substantially reducing the numbers of human pathogens, including *Campylobacter*, associated with the chicken skin when used under standard commercial operating conditions (24). Increasing the concentration of hyperchlorite does marginally increase its efficacy but reduces the quality of the end product, which is unacceptable to consumers.

Host-specific bacteriophage have previously been used in the treatment of *E. coli* infections in piglets (22) and more recently in *Enterococcus* (5) and *Vibrio* (7) infections. Their efficacy in experimental enteric and systemic infections has already been demonstrated. Other studies have shown that the

spoilage of beef by *Pseudomonas* spp. can be reduced by the application of phage (10). However, the ability of host-specific phage to reduce the presence of a food-borne pathogen which is not actively replicating on the surface of food has yet to be investigated.

The survival of the bacteriophage and its host independent of each other was determined on the surface of skin obtained from freshly sacrificed broiler chickens ( $n = 6$ ) reared to be free of *Campylobacter*. The cecal contents and skin of these birds were established as *Campylobacter* negative by the direct plating of samples onto modified charcoal cefoperazone deoxycholate agar (mCCDA; code LAB 112; supplement code X112; LabM, Lancashire, United Kingdom), a selective medium used in the isolation of thermophilic campylobacters. Each chicken was sacrificed at 30 days of age and then was manually plucked prior to skin removal. Sections of chicken skin (2 cm<sup>2</sup>) were then cut by using clean, flame-sterilized scissors and were individually transferred to sterile petri dishes.

The *Campylobacter* strain used, *C. jejuni* NCTC 12662 phage type 14 (*C. jejuni* PT14), was prepared by uniformly inoculating plates of Columbia blood agar (CBA; Columbia agar base, code CM331, supplemented with 7% [vol/vol] laked horse blood, code SR48; Oxoid Ltd., Basingstoke, United Kingdom) and incubating them in a microaerobic atmosphere (5% O<sub>2</sub>, 5% H<sub>2</sub>, 10% CO<sub>2</sub>, 80% N<sub>2</sub>) at 42°C for 24 h. The resulting growth was harvested into 20-ml volumes of sterile 10 mM MgSO<sub>4</sub> solution, and the cell density was adjusted to McFarland standard no. 3 (approximately 10<sup>9</sup> CFU per ml). All subsequent serial dilutions for the experimental inoculations were prepared in maximum recovery diluent (MRD; code CM733; Oxoid Ltd.). Bacteriophage  $\phi$ 2 was propagated on its host (*C. jejuni* NCTC 12662 PT14) by using the methods of Frost et al. (9) prior to adjusting the titer to 10<sup>8</sup> PFU per ml by dilution in SM buffer (50 mM Tris-Cl, pH 7.5, supplemented with 0.1 M NaCl, 8 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.01% gelatin [Sigma G-1393]). Each section of chicken skin was inoculated in triplicate with either 10<sup>6</sup> CFU of *C. jejuni* NCTC 12662 PT14 (estimated using McFarland turbidity standards and subsequently titered by dilution) or 10<sup>7</sup> PFU of bacteriophage  $\phi$ 2 (NCTC 12674, ACTC 35922-B2) suspension (titer determined

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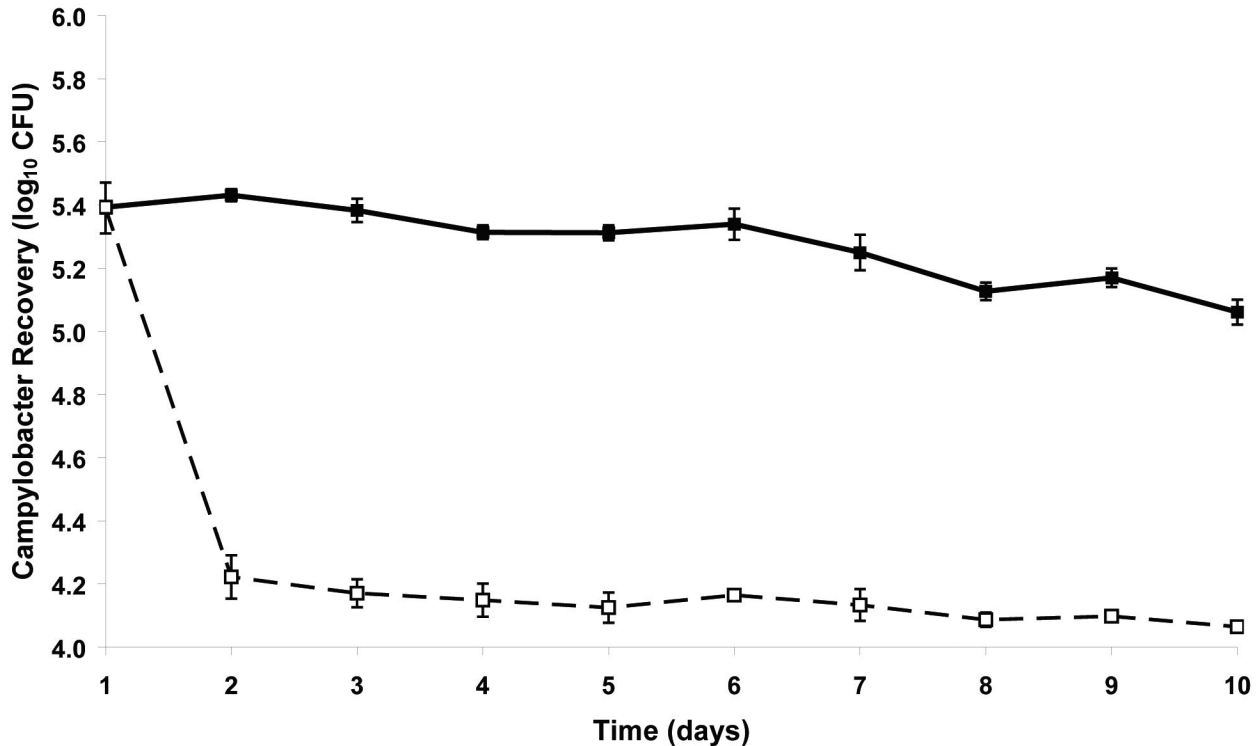


FIG. 1. Graph showing the recovery of *C. jejuni* NCTC 12662 from the surface of chicken skin inoculated with  $10^6$  CFU and stored at 4°C (■) and -20°C (□) over a 10-day period. Recovery is presented as the mean  $\log_{10}$  CFU  $\pm$  standard deviation.

on the *C. jejuni* NCTC 12662 host strain) for each sampling time point. The skin sections were then stored individually in sterile containers at either 4°C (fresh) or -20°C (frozen).

Sections of skin from each storage condition were removed for the recovery of both *Campylobacter* and bacteriophage, initially 1 h after inoculation (day 1) and then at 24-h intervals thereafter for a period of 10 days. *Campylobacters* were recovered by stomaching each chicken skin section in 20 ml of MRD for 5 min in sterile filter stomacher bags using a Seward Lab Blender 400. The stomachate was then serially diluted in MRD before spread plating 100- $\mu$ l volumes of each dilution onto the surface of mCCDA plates in triplicate. These plates were subsequently incubated in microaerobic conditions for 24 to 48 h before the enumeration of typical *Campylobacter* colonies. Representative colonies from each sample were subcultured onto CBA plates for 18 h before Gram staining and confirmation of typical *Campylobacter* cell morphology. Bacteriophage were recovered by stomaching the skin sections for 1 min in 20 ml of SM buffer in a sterile filter stomacher bag using a Seward Lab Blender 400. An aliquot of the stomachate (1 ml) was centrifuged at  $13,000 \times g$  for 3 min in a Centaur microcentrifuge to remove bacterial cells. Serial dilutions of the supernatant were used to inoculate a lawn of susceptible *Campylobacter* host cells (*C. jejuni* NCTC 12662) using a modification of the surface droplet technique (20) with enumeration performed according to the method of Miles and Misra (18). Bacterial lawns were prepared by adding 0.5 ml of a  $10^9$ -CFU/ml suspension of *C. jejuni* PT14 to 5 ml of molten NZCYM overlay agar (NZCYM broth [code 241510; Difco, Oxford, United Kingdom] with 0.6% bacteriological agar [Ox-

oid Agar No. 1, code L13]) and then pouring it over NZCYM base agar (NZCYM broth with 1% bacteriological agar) to ensure an even distribution. Plates were left to set for 20 min prior to the spotting of phage suspensions onto the surface. After inoculation of the phage the plates were left to dry for 20 min before incubation in microaerobic conditions at 42°C for 24 h and then were examined for the presence of plaques.

The recovery of *Campylobacter* NCTC 12662 from the surface of chicken skin is presented in Fig. 1. *Campylobacter* recovery at the initial time point was identical ( $\log_{10}$  5.4 CFU) for skin samples stored at 4 and -20°C. However, recovery from the fresh samples continued to gradually decline to reach a base of  $\log_{10}$  5.1  $\pm$  0.04 CFU at the end of the experiment. In contrast, the freeze-thaw treatment of the skin resulted in a fall in *Campylobacter* recovery to  $\log_{10}$  4.2  $\pm$  0.08 CFU on day 2 versus  $\log_{10}$  5.4  $\pm$  0.02 CFU for the fresh chicken skin at the same time point. On frozen skin, *Campylobacter* recovery was sustained at approximately the same level for the rest of the experiment with only a slight decline to  $\log_{10}$  4.1  $\pm$  0.01 CFU on day 10. Numerous studies have shown the prevalence and scale of *Campylobacter* contamination of retail poultry products (4, 14–16). Investigations into the survival of clinical *Campylobacter* isolates stored at different temperatures noted a  $\log_{10}$  3.0 CFU drop in viable cells upon freezing (8). Our data suggest this may not be the case for campylobacters on the surface of chicken skin, where the reduction was closer to  $\log_{10}$  1.0 or  $\log_{10}$  2.0 CFU for skin stored at 4 and -20°C, respectively. Considering these data, it is possible that chicken skin has a protective effect on the *Campylobacter* cells. Similar findings have been reported for other physical and chemical meth-

ods of removing *Campylobacter* from chicken carcasses (13). Studies with *Salmonella* also suggest that the chicken skin provides an environment that confers nonspecific protection to bacteria from potentially harmful treatments (13, 24). This is most likely due to the presence of feather follicles and folds on the skin surface that, along with oils and fats present, offer some protection from ice crystal formation, which is known to be a major cause of damage to cells undergoing the freezing process. Chicken carcasses have been reported to be contaminated with up to  $10^9$  CFU of *Campylobacter* (15). Since the infective dose of *Campylobacter* for humans is thought to be  $\leq 500$  cells (6), the information on the enhanced survival of *Campylobacter* on chicken skin under these conditions is of critical importance in food safety.

Bacteriophage  $\phi 2$  recovery from chicken skin stored at  $4^\circ\text{C}$  remained relatively constant at  $\log_{10} 6.7 \pm 0.14$  PFU of the initial inoculated titer ( $\log_{10} 7.0$  PFU) for the duration of the experiment, falling slightly to  $\log_{10} 6.4 \pm 0.1$  PFU at day 10. The recovery of phage inoculated on chicken skin stored at  $-20^\circ\text{C}$  was initially similar to recovery from skin stored at  $4^\circ\text{C}$  ( $\log_{10} 6.8 \pm 0.11$  PFU) in parallel with the pattern observed for its host, but following freeze-thaw the recovery fell to  $\log_{10} 6.1 \pm 0.2$  PFU, where it remained for the rest of the experiment. The fact that phage are able to survive on the surface of chicken skin for a period of at least 10 days when stored under either fresh or frozen conditions is important when considering the potential efficacy and environmental impact of phage therapy on animals that carry *Campylobacter*. Phage of *Campylobacter* along with their hosts can potentially survive on the retail product until well after its prescribed shelf life. A concurrent study has shown that *Campylobacter* phage are indeed able to survive and be isolated from retail poultry products.

To establish whether bacteriophage  $\phi 2$  bound nonspecifically to other *Campylobacter* species at the refrigerated temperature required for retail chicken storage ( $4^\circ\text{C}$ ), the phage was mixed with *Campylobacter coli* NCTC 12667, which is not susceptible to infection by this phage. A  $10^7$ -PFU suspension of  $\phi 2$  was mixed with a  $10^6$ -CFU/ml suspension of NCTC 12667 in MRD to give a multiplicity of infection (MOI) of 10. Triplicates of this suspension were stored statically at  $4^\circ\text{C}$ . *Campylobacter* and bacteriophage titers were recorded at 24-h intervals for 10 days. *Campylobacter*s were enumerated by using serial-dilution mCCDA spread plates as described previously. For bacteriophage enumeration, 1 ml of the suspension was centrifuged at  $13,000 \times g$  for 3 min in a Centaur microcentrifuge to remove bacterial cells. Serial dilutions of the supernatant were used to inoculate a lawn of susceptible *Campylobacter* host cells (*C. jejuni* PT14) for enumeration using the surface droplet technique described previously. This experiment was then repeated for  $\phi 2$  and susceptible host *C. jejuni* PT14 for comparison. There was no recorded fall in either the *Campylobacter* or bacteriophage titers when  $\phi 2$  was mixed with *C. coli* NCTC 12667 compared to titers of the controls. In contrast, when  $\phi 2$  was mixed with the susceptible host (*C. jejuni* PT14), falls of  $\log_{10} 0.8 \pm 0.1$  PFU/ml and  $\log_{10} 0.5 \pm 0.2$  CFU/ml were observed for phage and *Campylobacter* populations, respectively. There was no increase in phage titer over the 10-day course of the experiment with either *Campylobacter* species used. After the initial decrease in *Campylobacter* and phage titers were recorded, no further significant

TABLE 1. Matrix of *Campylobacter* and bacteriophage titers used for chicken skin inoculation

Amt. of <i>Campylobacter</i> inoculum (CFU)	Amt. of bacteriophage inoculum (PFU) and MOI <sup>a</sup>		
	$10^7$	$10^5$	$10^4$
$10^6$	10	0.1	0.01
$10^4$	1,000	10	1
$10^2$	100,000	1000	10

<sup>a</sup> The MOI for each phage-host combination is displayed.

changes in their numbers were observed for the remainder of the experiment at this temperature.

Sections of chicken skin ( $2\text{ cm}^2$ ), prepared as described above, were inoculated with combinations of different bacteriophage and host titers in order to determine the effect of phage application on the recovery of *Campylobacter* from artificially contaminated chicken skin stored at 4 and  $-20^\circ\text{C}$ . Each skin section was inoculated with a combination of phage and *Campylobacter* titers in the form of a simple 3 by 3 matrix to obtain MOIs ranging from 0.001 to 100,000 (Table 1). Each specific MOI combination was inoculated in triplicate for each sampling time point for each storage condition. Both phage and host were prepared for inoculation as described above. The skin was first inoculated with *C. jejuni* NCTC 12662, and following a 30-min drying period the phage ( $\phi 2$ ) was inoculated. A further 30-min period was allowed for the phage inoculum to dry before storage at  $4^\circ\text{C}$  (fresh) or  $-20^\circ\text{C}$  (frozen). The initial (day 1) samples were removed for *Campylobacter* and phage enumeration immediately following the final 30-min drying period and thereafter on days 3 and 5 for fresh samples and day 5 only for frozen samples. *Campylobacter* and bacteriophage populations were enumerated by using the methods described above. The significance of the effect of phage treatment on *Campylobacter* numbers was assessed with a one-tailed paired *t* test with a confidence interval of 95% using the Data Analysis Toolkit for Microsoft Excel XP. The recovery of *C. jejuni* from the surface of chicken skin following treatment with different titers of bacteriophage is presented in Table 2. *Campylobacter* recovery from controls inoculated with  $10^6$  and  $10^4$  CFU remain consistent for the course of the experiment, with recovery ranging from  $\log_{10} 5.2$  to  $\log_{10} 5.4$  CFU ( $10^6$ -CFU inoculation) and  $\log_{10} 3.2$  to  $\log_{10} 3.4$  CFU ( $10^4$ -CFU inoculation). With the lowest phage titer applied ( $10^3$  PFU) there was no significant fall in the numbers of *Campylobacter* recovered from fresh or frozen chicken skin compared to those of the control. However, in all cases of the highest-titer phage treatment ( $10^7$  PFU) there was a significant reduction ( $P < 0.0001$ ) in *Campylobacter* recovery at all sampling points, falling by  $\log_{10} 1.1$  to  $\log_{10} 1.2$  CFU ( $10^6$  CFU inoculation) and  $\log_{10} 1.1$  to  $\log_{10} 1.3$  ( $10^4$  CFU inoculation) versus those of their respective controls. The difference in recovery was even greater in the frozen chicken skin samples. When  $10^7$  PFU was applied, a reduction of  $\log_{10} 2.3$  and  $\log_{10} 2.5$  CFU compared to those of the controls ( $\log_{10} 4.1 \pm 0.6$  and  $\log_{10} 2.5 \pm 0.6$  CFU) was recorded for *Campylobacter* inoculations of  $10^6$  and  $10^4$  CFU, respectively. These reductions were also significant ( $P < 0.0001$ ) using a one-tailed paired *t* test.

TABLE 2. Recovery of *C. jejuni* NCTC 12662 from the surface of chicken skin treated with bacteriophage  $\phi 2$  and stored at 4°C<sup>a</sup>

Amt. of <i>Campylobacter</i> inoculum (CFU)	Amt. of bacteriophage inoculum (PFU) and day recorded									Control recovery at day:		
	10 <sup>7</sup>			10 <sup>5</sup>			10 <sup>3</sup>			1	3	5
	1	3	5	1	3	5	1	3	5	1	3	5
10 <sup>6</sup>	4.2 ± 0.2	4.2 ± 0.1	4.2 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	5.0 ± 0.1	4.9 ± 0.1	5.0 ± 0.1	5.3 ± 0.1	5.4 ± 0.1	5.2 ± 0.1
10 <sup>4</sup>	1.9 ± 0.1	2.1 ± 0.0	2.3 ± 0.0	3.4 ± 0.2	3.5 ± 0.0	3.1 ± 0.1	3.3 ± 0.1	3.4 ± 0.2	3.4 ± 0.1	3.1 ± 0.4	3.4 ± 0.2	3.4 ± 0.2
10 <sup>2</sup>	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 1.3	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 1.3	0.0 ± 0.0	0.0 ± 0.0

<sup>a</sup> Results are shown as log<sub>10</sub> CFU recovered ± standard deviations for different combinations of phage and host inocula recorded at days 1, 3, and 5.

The application of *Campylobacter*-specific bacteriophage in high enough titers to the surface of chicken skin inoculated with *Campylobacter* clearly reduced the number of recoverable cells. This reduction was consistent over the course of the experiment, with a fall in recovery of log<sub>10</sub> 1.0 CFU for inoculated skin stored at 4°C. A larger reduction would be desirable if this practice was to yield commercial benefit in the future as a sole control measure. However, in its present state it might be useful alongside other contamination control practices. There was a significantly greater reduction in *Campylobacter* recovery associated with the combination of freezing and phage treatment. Countries such as Iceland routinely freeze *Campylobacter*-positive carcasses in an attempt to reduce contamination, and recent studies suggest this approach is effective (23). Combining this freezing with phage treatment could result in further falls in *Campylobacter* prevalence on broiler carcasses. It is generally accepted that campylobacters do not replicate when incubated at 4°C. Our data support this, since the number of campylobacters which could be recovered from the surface of the skin fell over time. There was also no increase in bacteriophage numbers on any of the chicken skin samples inoculated with *Campylobacter*. Recovery of bacteriophage on all of the samples fell over time but did not differ significantly from that of the control. In the absence of active replication, the phage are unlikely to reduce the number of *Campylobacter* cells in situ. Consequently, the most likely explanation for the observed reduction in *Campylobacter* recovery is that upon inoculation a proportion of the phage successfully adsorb to the surface of the bacteria but do not replicate until the bacterium itself increases its metabolic activity. This is supported by the data showing there is no reduction in either bacteriophage or *Campylobacter* titer when the phage is mixed with a nonpermissive host, suggesting that nonspecific adsorption or lysis by the phage is not taking place. This has implications for using phage as decontaminating agents, as only populations of susceptible bacteria would be affected. Effective cocktails of broad-host-range phage would need to be used for this technique to be practically applicable.

In our experiments we have diluted the campylobacters recovered after phage treatment of the chicken skin before placing them under conditions that will allow growth. This procedure enabled us to enumerate the surviving bacteria. Under these circumstances any bacterial cells that are phage adsorbed will perish, but the phage from this burst will not easily disseminate to other host bacteria. However, in a situation where the phage-adsorbed bacteria are part of a larger, localized population of host bacteria, the phage burst will initiate new infection cycles. If the conditions permissive for growth were

indeed a human or animal gut, then the initial infective dose would be reduced with the prospect of further control by the phage on the growth of the host bacterium.

To determine if the campylobacters recovered after phage treatment were resistant to further phage infection, these isolates were subcultured onto CBA plates under microaerobic conditions at 42°C for 24 h and were used to prepare bacterial lawns on NZCYM plates. The lawns were then inoculated with serial dilutions of a  $\phi 2$  bacteriophage suspension, and plaques were enumerated using the surface droplet technique described above. The phage titers obtained from lawns of the recovered campylobacters were compared with titers determined with the initial strain. All of the campylobacters recovered from phage-treated chicken skin remained sensitive to  $\phi 2$ . The titers obtained on lawns of these bacteria did not differ significantly from those of the original strain. The recovered campylobacters were then examined for their genetic similarity to the inoculated strain by using pulsed-field gel electrophoresis (PFGE) to compare *Sma*I restriction fragments of genomic DNA using the method described by Ribot et al. (19). The PFGE macrorestriction profiles produced by *Sma*I digestion of the recovered *Campylobacter* strains were indistinguishable from that of the original inoculated strain. Adaptation of the host to become resistant to bacteriophage can readily be demonstrated in the laboratory, and this is postulated to be a major drawback in the use of phage as control agents in general. There was no evidence of resistance arising in the *Campylobacter* recovered after phage treatment in this study. The campylobacters recovered were confirmed as being identical to the inoculation strain by PFGE of *Sma*I restriction fragments of genomic DNA. However, in the absence of replication these studies do not rule out the generation of new mutational events selected posttreatment.

In conclusion, we have demonstrated the ability of a characterized NCTC *Campylobacter* and its bacteriophage predator to survive independently on the surface of chicken skin. Phage inoculated onto the surface of skin contaminated with *Campylobacter* exhibit a control effect even in the absence of host growth. Further development of this study could lead to the use of bacteriophage in connection with other measures to control chickens contaminated with *Campylobacter*.

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