Colonisation of a Phage Susceptible \textit{Campylobacter jejuni} Population in Two Phage Positive Broiler Flocks

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Abstract

The pathogens \textit{Campylobacter jejuni} and \textit{Campylobacter coli} are commensals in the poultry intestine and campylobacteriosis is one of the most frequent foodborne diseases in developed and developing countries. Phages were identified to be effective in reducing intestinal \textit{Campylobacter} load and this was evaluated, in the first field trials which were recently carried out. The aim of this study was to further investigate \textit{Campylobacter} population dynamics during phage application on a commercial broiler farm. This study determines the superiority in colonisation of a \textit{Campylobacter} type found in a field trial that was susceptible to phages in \textit{in vitro} tests. The colonisation factors, i.e. motility and gamma glutamyl transferase activity, were increased in this type. The clustering in phylogenetic comparisons of MALDI-TOF spectra did not match the ST, biochemical phenotype and phage susceptibility. Occurrence of \textit{Campylobacter jejuni} strains and phage susceptibility types with different colonisation potential seem to play a very important role in the success of phage therapy in commercial broiler houses. Thus, mechanisms of both, phage susceptibility and \textit{Campylobacter} colonisation should be further investigated and considered when composing phage cocktails.

Introduction

Phages are viruses that infect and kill bacteria. Even though they were widely used in the former Soviet Union, their use as antibacterial agents, has raised interest in the western world since antibiotic resistance has become a major problem in treating bacterial infections [1]. More recently, phages have been discussed for application in the food chain to control foodborne pathogens like \textit{Campylobacter} [2–4]. \textit{Campylobacter} is a Gram negative, motile bacterium and is the most frequent cause of zoonotic infections in developed countries with symptoms of mild diarrhoea, abdominal pain and fever. In 2011, there were 220,209 confirmed cases reported in the EU [5]. In about 1 out of 1000 cases, infections result in serious sequelae such as Guillain Barre Syndrome or Reactive Arthritis [6,7]. \textit{Campylobacter} is a commensal in birds, and broiler chickens are the main source for human infection [8,9]. \textit{Campylobacter jejuni} and \textit{C. coli} are the most frequently isolated species causing human infection. Combating \textit{Campylobacter} in primary production is considered to be effective for reducing human campylobacteriosis and different experiments have been carried out, confirming the efficacy of phages for reducing \textit{Campylobacter} load in chicken [10–14]. However, results have been highly variable in the onset and range of reduction and recently first field trials were performed to assess this issue under practical conditions. In two of three field trials a significant reduction was observed, but as in the other studies, the range of reduction varied widely and occurrence of different bacterial phenotypes may have influenced results of these studies [14].

Several studies have reported extensive biodiversity in \textit{C. jejuni} isolates populating broiler chickens [15–18] and more than one strain can coexist in one broiler flock at a time [19–22]. Results indicate that phenotypic differences occur in closely related strains of \textit{C. jejuni} and might be vital for the ability to colonise [23]. \textit{C. jejuni} has a largely non-clonal population structure and generates genetic diversity through intra- and interspecies recombination [24]. In contrast to other intestinal pathogens, host factors instead of classical virulence factors seem to play a major role for pathogenesis of human campylobacteriosis [25]. Published studies to date, indicate that intestinal colonisation of chickens and humans is a multifactorial process [26]. The motility, imparted by the polar flagella, seems to play an important role in this process but studies indicate that it is influenced by many metabolic factors [26–29]. Additionally, motility is considered to be an important pathogenic determinant of \textit{C. jejuni} [30]. Studies have demonstrated different expression patterns of flagella proteins between a poor and a robust coloniser strain in poultry [31,32]. The genes of proteins involved in the modification of the flagellum are located in hyper variable regions of the \textit{C. jejuni} genome [33]. While different studies found non-motile mutants exhibiting a decreased ability to enter cultural cells [34,35] other studies have found a number of mutants with measurable defects in interaction with cultural cells that retained full motility [36]. It remains unclear...
how exactly motility and flagella structure are involved in colonisation of the chicken intestinal tract [30,37,30]. Tests on *Campylobacter* metabolism revealed wide variability and impact of amino acid catabolism on colonisation of the intestine [39–41], which is reasonable when considering that this bacterium uses intermediates of the tricarboxylic acid cycle as an energy source [25]. GGT is an enzyme involved in amino acid catabolism, providing utilisation of glutamine and glutathione and cellular protection against reactive oxygen species [42]. It is reported to be required for persistent colonisation [28]. The presence of the *ggf* gene was reported to correlate directly with expression of the GGT activity [29,43].

While different studies have investigated the efficacy of applied phages, the development of resistance, and reduced susceptibility in vivo under a controlled environment [4,10–13,16,44–49] little is known about the interaction of applied phages with different *C. jejuni* strains and phenotypes under commercial conditions. It is well known that a diversity of strains occurs in different flocks and slaughter groups [17]. This can result in generation of non-susceptible isolates [12,32,48] and the modulation of genes and proteins that have an effect on colonisation of susceptible strains [47,50].

Interestingly, results of phage application trials using ≥3 week old birds by Loc-Carrillo et al. and Scott et al. [11,51] suggested that phage resistant isolates can be generated by chromosomal inversion, but on the other hand express reduced ability to colonise the broiler intestine. However, Carvalho and colleagues who used 1 week old chicks found no differences in the ability of resistant and susceptible phenotypes to colonise the chicken gut [12]. It is worth highlighting that those differences may be associated with the difference in the age of the birds in the trials and the method used to sample the *Campylobacter* isolates from the birds post treatment.

The present study aims to investigate the bacterial population dynamics of different *C. jejuni* strains and phenotypes in two phage positive flocks from one farm. Furthermore, it concentrates in investigating two factors which are considered to be important for *Campylobacter* colonisation in chicken intestine: motility and gamma glutamyl transpeptidase (GGT).

**Material and Methods**

**Field trials**

The farm under investigation in this study was located in the north of Germany. It included 8 sheds in 2 buildings. Each building included two floors and two sheds on each floor. The sheds of the two broiler flocks under investigation were located on different floors of one building. Birds of both flocks were of the same age and housed on the same day. The field trial on this farm was originally part of a larger study by Kittler et al. [14]. Samples were taken from two phage positive flocks: Flock 1, which contained broilers experimentally treated with phage; and Flock 2, which contained broilers that had been accidentally cross-contaminated with phage during the phage therapy trial. Application of phages in the field trials described by Kittler et al. [14] was performed according to German law. Formal approval of this study as an animal experiment was according to German law not necessary. However, the study was acknowledged by the Animal Welfare Committee of the University of Veterinary Medicine Hannover (competent ethics committee of the university). In accordance with German law permission to apply bacteriophages as feed additive was given by the competent authority (LAVES Az.: 41.3-63003-13/2011). Studies were performed on the farms R1 and M2. R1 was the farm under investigation in this study and is referred to throughout the manuscript as farm 1. Isolates of M2 (referred to throughout the manuscript as farm 2) were used for comparison of MLST analysis only. The owners gave their consent to participate in the study.

**Bacterial isolates**

All 668 *C. jejuni* isolates used in this study were derived from farm 1, during the third phage application field trial described by Kittler et al. [14]. The field trial was carried out in 2012, testing the effect of a phage cocktail to reduce *Campylobacter* in commercial broiler chickens. Briefly, in each of two broiler chicken flocks 9 individual samples of faeces or caecal content were taken for *Campylobacter* spp. and phage enumeration. The flocks were naturally colonised by *Campylobacter*. Samples were taken when birds were 31, 32, 35 and 38 days old.

Depending on the availability of single colonies, up to 100 isolates from each flock and sampling time were collected. If the samples of a sampling time contained less than 100 colonies, all available single colonies were isolated. This altogether resulted in 668 collected isolates from a total of 55 *Campylobacter* positive samples. Only 15 isolates from flock 1 at day 31 were isolated and >65 isolates for all other samplings. The isolates were stored in skimmed milk as described below. For further examination, isolates were grown in Preston selective broth (Oxoid, Germany) for 40 h under microaerobic conditions and subsequently cultivated on Karmali agar plates (Oxoid, Germany).

**Entry of phages**

As described in detail by Kittler et al. [14] phages were applied to flock 1 via drinking water after sampling of faeces for *Campylobacter* and phage detection from 31 day old birds. The phage cocktail consisted of 4 lytic, well characterised phages of the British phage typing scheme (NCTC 12672, 12678, 12674 and 12676). Flock 2 did not receive any phages. However, in flock 2 cross contamination occurred. Phages were found in the samples of this flock four days after phages had been applied in flock 1 [14].

**Detection of phages and *Campylobacter***

All samples were investigated for presence of phages and *Campylobacter* as previously described [14]. Presumptive *Campylobacter* colonies were picked from two plates containing <100 single colonies. They were cultivated on Karmali agar plates for 24 h and stored in skimmed milk at −80°C. Afterwards, they were examined for motility and typical cell morphology under the microscope and for positive catalase and oxidase reaction before storage.

**Multi Locus Sequence Typing**

A total of 18 selected isolates from the two flocks were characterised by MLST analysis according to the procedure outlined by Dingle et al. [32]. The amplification and sequencing primers were obtained from *C. jejuni* PubMLST webpage. The seven housekeeping genes loci *aspc* (aspartase), *gltA* (glutamine synthetase), *gldA* (citrate synthase), *gldC* (serine hydroxyl methyl transferase), *pgmA* (phosphor glucomutase), *ktl* (transketolase) and *uncA* (ATP synthase alpha subunit) were used. Sequence files were read, assembled, evaluated, aligned and compared to the reference set of alleles using BioNumerics 7.1 software (Applied Maths, Belgium). The 18 selected isolates were phylogenetically compared to 7 isolates of two other, similarly performed field trials [14] and to 314 strains isolated by members of the FIB-Zoo research network from different sources in Germany 2007–2011 [23]. Table 1 summarises the isolates and applied methods.
method previously described [53]. Briefly, 100 μl cocktail was tested, using a modification of the agar overlay control (no test suspension).

Parts at an overall concentration of log10 4 pfu/ml. The phage test suspension harboured all four cocktail phages in equal numbers, which were grown on MH blood plates (Oxoid, Germany) for 17 hours. The plates were incubated for 24 h and isolates were ranked in four susceptibility classes: Non- susceptible isolates without plaque formation; low susceptibility isolates (1–10 pfu/ 0.1 ml), medium susceptibility isolates (10–100 pfu/ 0.1 ml), high susceptibility isolates (100–1000 plaques/ 0.1 ml). C. jejuni NCTC 12662 was used for positive (log10 4 pfu/ml test suspension) and negative (log10 3–4 colonies) controls. These were prepared for each assay using the well characterised strain NCTC 12662 and 19660- 10 from our laboratory reference stock.

Phage susceptibility of Campylobacter isolates

Susceptibility of Campylobacter isolates to the phages in our cocktail was tested, using a modification of the agar overlay method previously described [53]. Briefly, 100 μl phage test suspension and 100 μl of the respective Campylobacter isolate (McFarland 3, equating log10 7–9 cfu/ml data not shown, in 10 mmol MgSO4) were added to 5 ml molten NZCYM Overlay agar (0.7% agar agar) and poured on plates of NZCYM base agar (1.5% agar agar). For preparing McFarland 3, Campylobacter was grown on MH blood plates (Oxoid, Germany) for 17 hours. The phage test suspension harboured all four cocktail phages in equal parts at an overall concentration of log10 4 pfu/ml.

Plates were incubated for 24 h and isolates were ranked in four susceptibility classes: Non- susceptible isolates without plaque formation; low susceptibility isolates (1–10 pfu/ 0.1 ml), medium susceptibility isolates (10–100 pfu/ 0.1 ml), high susceptibility isolates (100–1000 plaques/ 0.1 ml). C. jejuni NCTC 12662 was used for positive (log10 4 pfu/ml test suspension) and negative control (no test suspension).

Motility assay of Campylobacter isolates

Motility of all 668 isolates was tested by a method described previously by Gaynor et al. [34] with slight modification. Briefly, 1 μl McFarland 3 suspension of the isolate was stabbed into NZCYM agar plates containing 0.7% agar (Oxoid, Germany). McFarland 3 was prepared as stated above using cultures that had been incubated for 17 h. After 24 h incubation, the diameter of the colonised zone was measured.

GGT activity of Campylobacter isolates

All 668 isolates were tested for GGT activity by using Gamma-Glutamyl- Aminopeptidase Diatabs (Rosco Diagnostica, Taastrup, Denmark) according to the manufacturer’s instructions. The test was performed in 48 well microtiter plates. Positive- and negative-controls were prepared for each assay using the well characterised isolates (100–1000 plaques/ 0.1 ml).

Susceptibility isolates (10–100 pfu/ 0.1 ml), high susceptibility isolates (1–10 pfu/ 0.1 ml), medium susceptibility classes: Non- susceptible isolates without plaque formation; low susceptibility isolates (1–10 pfu/ 0.1 ml), medium susceptibility isolates (10–100 pfu/ 0.1 ml), high susceptibility isolates (100–1000 plaques/ 0.1 ml). C. jejuni NCTC 12662 was used for positive (log10 4 pfu/ml test suspension) and negative control (no test suspension).

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MALDI-TOF analysis

41 Representative isolates of all samplings and phenotypic groups were analysed by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS).

The procedure was adapted from the ‘ethanol/formic acid extraction’ validated by the manufacturer (Bruker Daltonics GmbH, Germany). Briefly, isolates were cultivated for 17 h and 3–4 colonies were suspended in tubes containing 150 μl bidistilled water and 450 μl absolute ethanol. Subsequently they were stored at −20°C. After thawing they were centrifuged at 2000 g, the liquid phase was removed and the pellet was resuspended in 25 μl formic acid (70%), mixed with 50 μl acetonitrile and was centrifuged. 1 μl of the supernatant was placed onto a polished steel MALDI target plate (Bruker Daltonik GmbH, Germany) at four MALDI target positions per strain. After drying at room temperature each sample was overlaid with 1 μl of matrix containing α-cyano-hydroxy-cinnamic acid in 2.5% trifluoroacetic acid and 50% acetonitrile in water (Bruker Daltonik GmbH). They were dried at room temperature and the MALDI-TOF MS measurement was performed using the Bruker Biotyper (Bruker Daltonik GmbH). Raw spectrum data were analysed using Bionumerics Software 7.1.

Biochemical phenotyping

The API Campy test system (Biomereix, France) was used for biochemical phenotyping of ≥250 representative isolates of all groups and samplings according to the manufacturer’s instructions. Briefly, an overnight culture of each isolate was prepared and suspended in NaCl and AUX medium. Subsequently, microtubes of the API Campy strip were charged and incubated. The reactions were read according to the manufacturer’s instructions and analysed using Bionumerics Software 7.1.

Data analysis

SAS 9.3 software was used for statistical analysis. For detecting significant differences in Campylobacter counts, the Wilcoxon rank sum test was used.
Table 2. GGT activity, MLST (ST), clonal complex (CC) and phage susceptibility of representative isolates of the investigated field trial.

| Isolate | Species | GGT activity | ST       | CC           | Phage susceptibility |
|---------|---------|--------------|----------|--------------|---------------------|
| Flock 1 |         |              |          |              |                     |
| 3–554   | C.jejuni| –            | 6836     | ST-1034 complex | high               |
| 3–102   | C.jejuni| –            | 6836     | ST-1034 complex | none               |
| 3–827   | C.jejuni| –            | 6836     | ST-1034 complex | none               |
| 3–381   | C.jejuni| –            | 6836     | ST-1034 complex | low                |
| 3–353   | C.jejuni| –            | 4755     | ST-1034 complex | high               |
| 3–310   | C.jejuni| –            | 4755     | ST-1034 complex | none               |
| 3–840   | C.jejuni| –            | 4755     | ST-1034 complex | high               |
| 3–891   | C.jejuni| –            | 4755     | ST-1034 complex | none               |
| 3–540   | C.jejuni| –            | 4755     | ST-1034 complex | none               |
| 3–003   | C.jejuni| –            | 4755     | ST-1034 complex | high               |
| 3–013   | C.jejuni| +            | 4755     | ST-1034 complex | low                |
| 3–058   | C.jejuni| –            | 4755     | ST-1034 complex | none               |
| 3–722   | C.jejuni| –            | 4755     | ST-1034 complex | none               |
| 3–005   | C.jejuni| –            | 4755     | ST-1034 complex | none               |
| 3–714   | C.jejuni| –            | 4755     | ST-1034 complex | high               |
| 3–791   | C.jejuni| –            | 4755     | ST-1034 complex | high               |
| 3–032   | C.jejuni| –            | 4755     | ST-1034 complex | none               |
| 3–721   | C.jejuni| –            | 4755     | ST-1034 complex | none               |

*Flock 1.

Table 2. GGT activity, MLST (ST), clonal complex (CC) and phage susceptibility of representative isolates of the investigated field trial.

For evaluating susceptibility distribution and GGT activity the Chi-square test or Fisher-Yates-test were used and statistical significant differences in motility were detected using the t-test or Wilcoxon-rank sum-test.

MLST profiles, MALDI-TOF spectra and Biochemical profiles of isolates were compared by using the Minimum Spanning Tree plugin, and the cluster analysis tool of the BioNumerics 7.1 software.

Results

A total of 668 C. jejuni isolates were collected from two flocks before and after Campylobacter-phage application on a commercial broiler farm. The isolates were analysed for phage susceptibility, motility and biochemical phenotype. For phylogenetic comparison of selected isolates MLST sequence type (ST) and clonal complex (CC) analysis was carried out.

MLST analyses of 18 selected isolates (9 isolates from each flock of the investigated farm 1, thus including isolates of all samplings) revealed two STs that differed in two of seven MLST loci: 14 isolates belonged to ST 4755, whereas a new ST 6836 was identified. Results of comparing MLST profiles of field trial isolates and the isolates analysed by Gripp et al. are shown in Fig 1 B. The two STs of the investigated farm 1 isolates showed a minimal difference in their allelic profile compared to the ST 1709 isolates analysed by Gripp et al. [23]. The ST 1709 isolates derived from poultry meat in 2008 (Isolate FBI 04313; pubmlst.org) and from a laying hen 2009 (Isolate FBI 04343; pubmlst.org) [23].

The 668 isolates were further examined. Two phenotypes differing in the presence or absence of GGT activity were identified. Results of Campylobacter and phage detection in samples and frequency of GGT activity in both flocks are shown in Table 3. The isolates with GGT activity were significantly more often susceptible to phage infection than isolates displaying no GGT activity (p<0.0001, N = 668, Table 4). However, as displayed in Table 4, 38 isolates did not express these most frequent phenotypic combinations of GGT activity and phage susceptibility (Table 4, no GGT activity and phage susceptibility or GGT activity and no phage susceptibility). These isolates originated from all samplings of the field trial.

In flock 1 a phage cocktail was applied when birds were 31 days old [14]. The results displayed in Table 3 indicate that phages spread rapidly in the flock until they were found in 8 out of 9 samples after four days. Results of Campylobacter detection during the same period (Table 3) showed one positive sample (detection limit 50 cfu/g) when birds were 31 days old before phage application. Results of subsequent testings indicated a rapid spread of Campylobacter until all samples harboured Campylobacter after 10
days, suggesting colonisation occurring despite the presence of phages (Table 3; Flock 1).

Distribution of GGT activity among isolates at different times is shown in Table 3. Isolates with GGT activity were isolated mainly after phage application and results indicate a rapid spread and improved colonisation compared to isolates without GGT activity (Table 3; Flock 1). Looking at Fig. 2; A, non- susceptible isolates only were isolated from most samples on the first two samplings, changing over to both, susceptible and non- susceptible isolates, in all samples at the third sampling. Thus, results indicated that the susceptible isolates spread rapidly while non- susceptible isolates Table 3.

**Figure 1. Minimum spanning tree of MLST allelic profiles depicting the clustering of the sequence types.** Red dots: isolates presented in this paper (field trial farm 1*) Isolates for comparison: isolates of farm 2 from 2011 (yellow dots) and 2012 (orange dots), isolates published by Gripp et al. 2011 (green dots) Clonal complexes (maximum two allele difference between neighbouring STs) are indicated by coloured shading around circles. Most important STs and CCs are indicated by numbers. *including isolates from Kittler et al. 2013 (18 farm 1 isolates, 7 farm 2 isolates). doi:10.1371/journal.pone.0094782.g001

**Table 3. Phages and *Campylobacter* isolated on farm 1 and distribution of gamma glutamyl transferase activity.**

| Age of birds (days) | Phages | *Campylobacter* | Number of isolates without GGT activity (%) | Number of isolates with GGT activity (%) |
|---------------------|--------|-----------------|-------------------------------------------|---------------------------------------|
| Flock 1             |        |                 |                                           |                                       |
| 31                  | 0/9    | 1/9             | 15 (100)                                  | 0 (0)                                 |
| 32                  | 2/9    | 7/9             | 96 (96)                                   | 4 (4)                                 |
| 35                  | 8/9    | 7/9             | 40 (40)                                   | 59 (60)                               |
| 38                  | 8/9    | 9/9             | 9 (13)                                    | 58 (87)                               |
| Flock 2             |        |                 |                                           |                                       |
| 31                  | 0/9    | 5/9             | 5 (5)                                     | 91 (95)                               |
| 32                  | 0/9    | 8/9             | 6 (6)                                     | 92 (94)                               |
| 35                  | 8/9    | 9/9             | 0 (0)                                     | 99 (100)                              |
| 38                  | 7/9    | 9/9             | 15 (16)                                   | 79 (84)                               |

A significant decrease of log$_{10}$ 1.8 cfu/g in mean *Campylobacter* counts took place in the investigated flock 2 from day 35 to day 38 (p = 0.00078). All samples were faecal samples except for the last sampling in the flocks which were caecal samples from the slaughter- house. Detection limit was 50 cfu or pfu/g, respectively for phages and *Campylobacter* in all samples.

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Table 4. Susceptibility of Campylobacter jejuni isolates with and without gamma glutamyl transferase (GGT) activity on farm 1.

|                | No GGT activity | GGT activity |
|----------------|-----------------|--------------|
| Non-susceptible| 161*            | 33*          |
| Susceptible    |                 |              |
| low            | 1               | 23           |
| medium         | 8               | 38           |
| high           | 16              | 388          |
| total          | 25*             | 449*         |

*Chi-square test p<0.0001.

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discerned (Fig. 2; A). As displayed in Table 2, neither GGT activity nor phage susceptibility coincided with a certain MLST profile.

In flock 2, entry of phages occurred due to unintended cross-contamination and horizontal transmission, in contrast to phage application in flock 1 [14]. While no phages were detected from fecal samples tested at day 32, three days later 8 out of 9 samples were tested positively (Table 3; Flock 2). More than 50% of the samples tested positive for Campylobacter when birds were 31 days old and subsequent spreading was observed until all samples were Campylobacter positive 4 days later (detection limit 50 cfu/g).

Isolates with and without GGT activity were present at day 31 in flock 2 but 100% of tested isolates showed GGT activity four days later. However, at the last sampling, isolates without GGT activity reappeared when numbers of Campylobacter were reduced (Table 3; Flock 1). In this flock most samples harboured both susceptible and non-susceptible isolates at the beginning of observation (Fig. 2; B), but only susceptible isolates were isolated one day later. However, non-susceptible isolates reappeared six days later, at the last sampling (Fig. 2; B).

To determine if alteration of susceptibility or GGT activity correlated with changes in motility of the isolates, a motility assay was performed. Results are shown in Figure 3. Isolates with GGT activity showed a significantly higher motility than isolates without GGT activity (p<0.0001). Isolates were ranked in four susceptibility classes and even the lowest susceptibility class with 100 fold reduced susceptibility (1–10 plaques compared to 100–1000 in fully susceptible control) expressed a significantly higher motility compared to the non-susceptible isolates (Fig. 3 B; p = 0.0288 for low susceptibility; p = 0.0060 for medium susceptibility; p<0.0001 for high susceptibility).

Discussion

In this study the dynamics of Campylobacter colonisation in presence of phages in two broiler flocks was investigated. MLST, MALDI-TOF and biochemical analysis, phage susceptibility and motility as well as GGT activity were examined.

The results of the present study indicate that a phage susceptible C. jejuni subpopulation with increased motility and GGT activity could overgrow a non-susceptible subpopulation in the presence of phages. Susceptibility of the isolates was only tested in vitro. Even though we used standard test methods for phage susceptibility, we cannot rule out that differences between in vitro and in vivo susceptibility existed. A reduced ability of phages to kill in-vitro-susceptible Campylobacter in vivo was reported in another study [11]. Nevertheless, phage replication occurred during the trials and is a sign of sufficient concentrations of susceptible Campylobacter. According to pharmacokinetic experimental and modelling approaches of Cairns et al. [46] minimum concentrations of approx. log10 4 cfu/g of non-culturable Campylobacter isolates must have been present, if phages did not replicate in the susceptible Campylobacter that were isolated. Reappearance of non-susceptible isolates in flock 2 occurred when phages reached high concentrations and Campylobacter counts were reduced in this flock [14]. Even though no significant Campylobacter reduction occurred in flock 1, phage replication up to a mean of log10 5 pfu/g caecal content was observed [14]. These findings suggest that Campylobacter may have been under selective pressure of phage induced lysis in this trial. These results support the findings of Loc Carrillo et al. and Scott et al. [11,47,51] who found that a resistant phenotype had fitness costs and was rapidly dominated by a susceptible phenotype in vivo. Scott et al. [47] found dominance of the susceptible strain only when phages were absent, which is in contrast to our findings (Table 3). However, Scott et al. [47] found a horizontal gene transfer of >112 kb to be responsible for change of MLST ST and loss of susceptibility while the metabolic features of the dominant strain remained unclear. Nonetheless, in vivo experiments might not reflect conditions in commercial broiler houses.

MLST analysis of the 18 selected isolates found presence of STs 4755 and 6836. Both STs belonged to the ST-1034 CC. They were found to differ in only one allele compared to each of these STs [23], demonstrating that the isolates of the investigated field
trail were closely related to different isolates from other poultry sources. Isolates from other field trials revealed unrelated STs (Fig. 1). This finding is in accordance with Bull et al. [54] who found unrelated STs being present in one flock. Different susceptibility classes and GGT activity were found for isolates having the same ST (Table 2). These results are in contrast to Scott et al. [47] who found resistance to be associated with change of MLST locus gtm allelic profile. All 668 isolates were tested for phage susceptibility and were subdivided into four levels of susceptibility: Non-susceptible, low susceptible, medium susceptible and high susceptible correspondent to the number of observed plaques (10 and 100 fold reduced for medium and low susceptibility compared to control NCTC 12662). In both flocks an increase of phage susceptible isolates was observed during the trial. When numbers of Campylobacter were reduced in flock 2 [14], non-susceptible isolates reappeared (Fig. 2). GGT activity was found to be correlated with phage susceptibility (p<0.0001, Table 4). Due to the fact that some isolates displayed phage susceptibility but no GGT activity or vice versa (Table 4), results indicate that there is no direct link between susceptibility and GGT activity. It is more likely that the GGT active phenotype is linked to other mechanisms which determine phage resistance. We explicitly looked for new STs among the isolates that displayed unusual GGT- phage susceptibility phenotypes (Table 4). These isolates are thus overrepresented in Table 2. However, they displayed the same STs as the frequent phenotypes did. As they occurred in low numbers in all samplings, they do not seem to play a major role in the population dynamics of this field trial. In order to detect additional strains or subtype- clusters we looked for clustering of isolates using BioNumerics 7.1 analysis for MALDI-TOF raw spectra and ApiCampy phenotypes. Isolates from all samplings and all possible GGT- phage susceptibility phenotypes were included in these tests. The typing methods applied were performed consecutively, i.e. when MLST and ApiCampy did not reveal enough detail, MALDI-TOF was additionally applied. Thus the isolates selected for each test varied slightly. Clustering by ApiCampy was not reliable as was indicated by branch quality error flags (data not shown). Cluster analysis of raw spectra using peak based pearsson correlation indicated two reliable clusters (Fig. S1). However, they included different STs and all combinations of GGT-phage susceptibility phenotypes (Fig. S1). These results suggest that no additional strain or subtype was isolated. In recent studies GGT was found to be important for the colonisation of chickens and was associated with severe human cases [28,55,56]. To investigate further reasons for the superiority in colonisation of the phage susceptible GGT positive phenotype, we investigated motility of all isolates. Motility is considered to be important for Campylobacter colonisation [25,27]. An increased motility of the susceptible phenotype was found (Fig. 3), which could explain why susceptible C. jejuni isolates replaced non-susceptible isolates in the presence of phages, since GGT activity and increased motility might promote colonisation potential of this predominant phenotype. Thus, the results of previous in vivo studies were confirmed which found reduced motility of phage resistant phenotypes [51,57] and sensitive revertants overgrowing the resistant phenotype [47]. In contrast, Carvalho at al. [12] found no differences in colonisation potential of resistant and susceptible isolates.

Different mechanisms have been reported that influence phage susceptibility of Campylobacter. These mechanisms can cause reversible or non-reversible genetic or metabolic changes and can thus lead to alteration in genotype or phenotype [16,47,48,50,51,57,58]. While some of these alterations might be reflected in MLST allelic profile, others might not [55,59]. MLST STs and phage susceptibility did not coincide in our trial, thus demonstrating that the mechanism responsible for change of the MLST housekeeping genes gtm and ApyA did not influence susceptibility of the isolates. In contrast, the presence of susceptible and non-susceptible isolates of both STs implies that other, very variable mechanisms might be responsible for the change of susceptibility and correlation with motility and GGT activity. Possible interpretations of this phenomenon include phase variation and pleiotropic effects as reported for other phage-resistant bacteria [60-63]. Structural features such as the flagellum and CPS are known to undergo phase variation [50,64] and might contain receptor molecules necessary for phage adsorption [65]. Thus, in our study phase variation might have occurred and might have had pleiotropic effects on GGT activity. Furthermore, two separate mechanisms might have mediated resistance to different cocktail phages and have had effects on different metabolic

Figure 3. Mean motility of C. jejuni differed significantly between isolates with diverging GGT activity and susceptibility. Motility was tested on semisolid agar inoculating drops of 1 μl McFarland 3 (approx. log10 8 cfu/ml) suspension. Differences of p<0.05 were considered as significant. Stars on vertical lines represent level of significance (**:P<0.0001; ***:P<0.001; *:P<0.05). Error bars represent standard error of the mean. (A) Comparison of mean motility in isolates with and without GGT activity (B) Comparison of mean motility of isolates exhibiting different phage susceptibility classes.
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features of the isolates. Further research is needed for a better understanding of underlying mechanisms.

Despite successful colonisation of the susceptible phenotype, Kutler et al. [14] demonstrated reduction of Campylobacter concentration in chicken caeca of the second flock. Reduced numbers of Campylobacter in phage positive flocks were also reported previously by Atterbury et al. [45] in an epidemiological investigation.

Supporting Information

**Figure S1 Dendrogram of MALDI-TOF mass spectra and MLST sequence and phenotypes of Campylobacter jejuni isolates.** Sequence and phenotypes of isolates are not consistent with clustering of mass spectra. The dendrogram was generated using peak based pearson correlation and UPGMA algorithm in BioNumerics 7.1. Error flags indicate branch quality by standard deviation associated with each cluster. Error flags which don’t overlap indicate consistent clustering (green error flag). While overlapping error flags indicate non-consistent clusters (red error flags). Triangles display GGTT activity (white triangle: no GGTT activity, black triangle: GGTT activity). Hexagons display phage susceptibility (white hexagon: non susceptible, black hexagon: susceptible). * Test result with low sensitivity: no distinct GGTT phenotype. n.d. ST of this isolate was not tested. (TIF)

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**Author Contributions**

Conceived and designed the experiments: SK SF AA GG GK. Performed the experiments: SK AA. Analyzed the data: SK AA GK. Contributed reagents/materials/analysis tools: GK. Wrote the paper: SK SF AA GG GK.

**References**

1. Kutela M, Adamia R (2010) Bacteriophages as potential new therapeutics to replace or supplement antibiotics. Trends Biotechnol 28: 591–595.
2. Goodridge LD, Bish A (2011) Phage-based biocontrol strategies to reduce foodborne pathogens in foods. Bacteriophage 1: 130–137.
3. Greer GG (2005) Bacteriophage Control of Foodborne Bacteria. J Food Prot 68: 1102–1111.
4. Janez N, Loc-Carrillo C (2013) Use of phages to control Campylobacter spp. J Microbiol Methods 95: 65–73.
5. EFSA (2013) The European Union Summary Report on Trends and Sources of foodborne pathogens in foods. Bacteriophage 1: 130–137.
6. El-Shibiny A, Scott A, Timms A, Metawea Y, Connerton P, et al. (2009) Enteric Campylobacter purging its secrets? Pediatr Res 55: 3–12.
7. Nachamkin I, Allos BM, Ho T (1998) Campylobacter species and Guillain-Barre syndrome. Clin Microbiol Rev 11: 555–567.
8. Hermans D, Pasmans F, Messens W, Martel A, Van Immerseel F, et al. (2012) Poultry as a host for the zoonotic pathogen Campylobacter jejuni. Vector Borne Zoonotic Dis 12: 89–98.
9. Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, et al. (2008) Tracing the source of campylobacteriosis. PLoS Genet 4: e1000203.
10. Klein G, Beckmann L, Vollmer HM, Bartelt E (2007) Predominant strains of Campylobacter jejuni in broiler chickens. Appl Environ Microbiol 73: 6534–6538.
11. Carvalho CM, Gosson BW, Hallbeke DE, Santos SB, Hayes CM, et al. (2010) The in vivo efficacy of two administration routes of a phage cocktail to reduce numbers of Campylobacter coli and Campylobacter jejuni in broilers. BMC Microbiol 10: 232.
12. El-Shibiny A, Scott A, Timans A, Metauxa Y, Connerton P, et al. (2009) Application of a group II Campylobacter bacteriophage to reduce strains of Campylobacter jejuni and Campylobacter coli colonizing broiler chickens. J Vet Microbiol 109: 275–283.
13. LOC Carrillo C, Atterbury RJ, el-Shibiny A, Connerton PL, Dillen E, et al. (2005) Bacteriophage therapy to reduce Campylobacter jejuni colonization of broiler chickens. Appl Environ Microbiol 71: 6534–6538.
14. Kittler S, Fischer S, Abulmasood A, Glunder G, Klein G (2013) Effect of bacteriophage application on Campylobacter jejuni loads in commercial broiler flocks. Appl Environ Microbiol 79: 7525–7533.
15. El-Shibiny A, Connerton PL, Connerton IF (2005) Bacteriophage therapy to reduce Campylobacter jejuni colonization of broiler chickens. Appl Environ Microbiol 71: 6534–6538.
16. Connerton PL, Loc Carrillo CM, Swift G, Dillen E, Scott A, et al. (2004) Longitudinal study of Campylobacter jejuni bacteriophages and their hosts from broiler flocks. Appl Environ Microbiol 70: 3877–3883.
17. Lienau JA, Ellerbroek L, Klein G (2007) Tracing flock-related Campylobacter jejuni clones from broiler farms through slaughter to retail products by pulsed-field gel electrophoresis. Arch Lebensmittelhyg 70: 536–542.
18. Klein G, Beckmann L, Vollmer HM, Bartelt E (2007) Predominant strains of thermophilic Campylobacter spp. in a German poultry slaughterhouse. Int J Food Microbiol 117: 324–329.
19. El-Adawy H, Hotzel H, Tomaso H, Neubauer H, Taboada EN, et al. (2013) Detection of genetic diversity in Campylobacter jejuni isolated from a commercial turkey flock using flaA typing, MLST analysis and microarray assay. PLoS One 6: e25382.
20. Alter T, Weber RM, Hamedy A, Glunder G (2011) Carry-over of thermophilic Campylobacter jejuni from sequential and adjacent poultry flocks. Vet Microbiol 147: 90–95.
21. Ridley AM, Morris VK, Cawkwell SA, Fälls-Verden J, Harris JA, et al. (2011) Longitudinal molecular epidemiological study of thermophilic campylobacters on one conventional broiler chicken farm. Appl Environ Microbiol 77: 98–107.
22. Zweifel C, Schue KD, Krell M, Renegi F, Stephan R (2008) Occurrence and genotypes of Campylobacter in broiler flocks, other farm animals, and the environment during several rearing periods on selected poultry farms. Int J Food Microbiol 125: 182–187.
23. Gripp E, Hlahla D, Dideolt X, Kops F, Mauritshuis S, et al. (2011) Closely related Campylobacter jejuni strains from different sources reveal a generalist rather than a specialist lifestyle. BMC Genomics 12: 584.
24. de Boer P, Wagenaar JA, Achterberg RP, van Putten JP, Schools LM, et al. (2002) Generation of Campylobacter jejuni genetic diversity in vivo. Mol Microbiol 44: 351–359.
25. Dastl JT, Tarre AM, Lugert R, Zaumter AE, Gross U (2010) Campylobacter jejuni: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. Int J Med Microbiol 300: 205–211.
26. Hermans D, Van Deun K, Martel A, Van Immerseel F, Messens W, et al. (2011) Colonization factors of Campylobacter jejuni in the chicken gut. Vet Res 42: 02.
27. Hendriksen DR, DiSaia VJ (2004) Identification of Campylobacter jejuni genes involved in commensal colonization of the chick gastrointestinal tract. Mol Microbiol 52: 471–484.
28. Barnes IH, Bagnall MC, Browning DD, Thompson SA, Manning G, et al. (2007) Gamma-glutamyl transpeptidase has a role in the persistent colonization of the avian gut by Campylobacter jejuni. Microb Pathog 43: 198–207.
29. Rathbun KM, Hall JE, Thompson SA (2009) Cj0596 is a periplasmic peptidyl prolyl cis-trans isomerase involved in Campylobacter jejuni motility, invasion, and colonization. BMC Microbiol 9: 160.
30. Grant CC, Konkel ME, Cieplak W Jr., Tompkins LS (1993) Role of flagella in adhesion, internalization, and translocation of Campylobacter jejuni in nonpolarized and polarized epithelial cell cultures. Infect Immun 61: 1764–1771.
31. Hett KL, Stiatus A, Anschutz TM, Knaas RL, Neal BS (2000) Genomic differences between Campylobacter jejuni isolates identify surface membrane and flagellar function gene products which potently affect colonization of the chicken intestine. Funct Integr Genomics 3: 407–420.
32. Carrillo-CD, Taboada E, Nash JH, Lanthier P, Kelly J, et al. (2004) Genome-wide expression analyses of Campylobacter jejuni NCTC11168 reveals coordinate regulation of motility and virulence by phfA. J Biol Chem 279: 20327–20338.
33. Young KT, Davis LM, Dirsa VJ (2007) Campylobacter jejuni: molecular biology and pathogenesis. Nat Rev Microbiol 5: 663–679.
34. Gaynor EC, Cawthraw S, Manning G, MacKichan JK, Falkow S, et al. (1994) Isolation of motile and non-motile insertion mutants of Campylobacter jejuni: the role of motility in adherence and invasion of eukaryotic cells. Mol Microbiol 13: 255–265.
35. Novik V, Hofreuter D, Galan JE (2003) Identification of Campylobacter jejuni genes involved in its interaction with epithelial cells. Infect Immun 71: 3540–3553.
51. Scott AE, Timms AR, Connerton PL, Loc Carrillo C, Adzfa Radzum K, et al. (2007) Genomic dynamics of Campylobacter jejuni in response to bacteriophage predation. PLoS Pathog 3: e119.

52. Dingle KE, Colles FM, Wareing DR, Ure R, Fox AJ, et al. (2001) Multilocus sequence typing system for Campylobacter jejuni. J Clin Microbiol 39: 14–23.

53. Fischer S, Kitler S, Klein G, Glünder G (2013) Microplate-Test for the Rapid Determination of Bacteriophage-Susceptibility of Campylobacter Isolates-Development and Validation. PLoS ONE 8: doi:10.1371/journal.pone.0053899. PubMed: 23349761.

54. Bull SA, Allen VM, Domingue G, Jorgensen F, Frost JA, et al. (2006) Sources of Campylobacter spp. colonizing housed broiler flocks during rearing. Appl Environ Microbiol 72: 643–632.

55. Zauner AE, Oikh C, Taylor AM, Lugert R, Gross U (2012) Epidemiological association of Campylobacter jejuni groups with pathogenicity-associated genetic markers. BMC Microbiol 12: 171.

56. Ahmed IH, Manning G, Wasenmaier TM, Cawthraw S, Newell DG (2002) Identification of genetic differences between two Campylobacter jejuni strains with different colonization potentials. Microbiology 148: 1203–1212.

57. Coward C, Grant AJ, Swift G, Philp J, Towler R, et al. (2006) Phase-variable surface structures are required for infection of Campylobacter jejuni by bacteriophages. Appl Environ Microbiol 72: 4638–4647.

58. Abedon ST (2012) Bacterial ‘immunity’ against bacteriophages. Bacteriophage 2: 50–54.

59. Taboada EN, Mackinnon JM, Luebbert CC, Gannon VP, Nash JH, et al. (2008) Comparative genomic assessment of Multi-Locus Sequence Typing: rapid accumulation of genomic heterogeneity among clonal isolates of Campylobacter jejuni. BMC Evol Biol 8: 229.

60. Chatterjee AN, Mirelman D, Singer HJ, Park JT (1969) Properties of a novel pleiotropic bacteriophage-resistant mutant of Staphylococcus aureus. J Bacteriol 100: 846–853.

61. Heierson A, Siden I, Kivaisi A, Boman HG (1986) Bacteriophage-resistant mutants of Bacillus thuringiensis with decreased virulence in pupae of Hyalophora cecropia. J Bacteriol 167: 18–24.

62. Wandesman C, Moreno F, Schwartz M (1980) Pleiotropic Mutations Rendering Escherichia-Coli-K-12 Resistant to Bacteriophage Tp1. J Bacteriol 143: 1374–1383.

63. Blakemore TR, Sadowsky RB (1983) Congugal Transfer from Streptococcus-Lactis Me2 of Plasmids Encoding Phage Resistance, Nisin Resistance and Lactose-Fermenting Ability - Evidence for a High-Frequency Conjugative Plasmid Responsible for Abortive Infection of Virulent Bacteriophage. J Gen Microbiol 131: 1531–1541.

64. Park SF, Purdy D, Leach S (2000) Localized reversible frameshift mutation in the flhA gene confers phase variability to flagellin gene expression in Campylobacter coli. J Bacteriol 182: 207–210.

65. Lindberg AA (1973) Bacteriophage receptors. Annu Rev Microbiol 27: 205–241.