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Survival of *Campylobacter jejuni* in Water: Effect of Grazing by the Freshwater Crustacean *Daphnia carinata* (Cladocera)

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Environmental studies of the human-pathogenic bacterium *Campylobacter jejuni* have focused on linking distributions with potential sources. However, in aquatic ecosystems, the abundance of *C. jejuni* may also be regulated by predation. We examine the potential for grazing by the freshwater planktonic crustacean *Daphnia carinata* to reduce the survival of *C. jejuni*. We use a system for measuring grazing and clearance rates of *D. carinata* on bacteria and demonstrate that *D. carinata* can graze *C. jejuni* cells at a rate of 7% individual^{-1} h^{-1} under simulated natural conditions in the presence of an algal food source. We show that passage of *C. jejuni* through the *Daphnia* gut and incorporation into fecal material effectively reduces survival of *C. jejuni*. This is the first evidence to suggest that grazing by planktonic organisms can reduce the abundance of *C. jejuni* in natural waters. Biomanipulation of planktonic food webs to enhance *Daphnia* densities offers potential for reducing microbial pathogen densities in drinking water reservoirs and recreational water bodies, thereby reducing the risk of contracting water-borne disease.

Thermophilic bacteria of the genus *Campylobacter* are the most commonly reported bacterial cause of gastroenteritis in industrialized countries (10), and account for ca. 10% of all diarrhea worldwide (1). Of the gram-negative, spiral campylobacters, *Campylobacter jejuni* alone can attack otherwise healthy individuals, making it the most common cause of human campylobacteriosis (23, 32, 36). A number of outbreaks of campylobacteriosis have been linked to nine apparently contaminated and insufficiently treated drinking water supplies (20, 34, 39), however, in these instances, *C. jejuni* has rarely been isolated from the suspected water supplies. Although human sewage and animal fecal matter are the most likely sources of environmental contamination by campylobacters, clear and direct links between these potential sources and campylobacters in drinking or recreational waters have rarely been established, owing partly to the complex behavior of campylobacters once in the aquatic environment.

Campylobacter replication is thought to occur almost exclusively in the intestinal tract of mammalian and avian hosts (2, 16, 31). Once excreted into aquatic environments, campylobacters can undergo physiological change to a viable, nonculturable state (5, 6, 22, 30, 35, 39) and, in both the nonculturable and the culturable states, they can persist in water and be transported from their sources. Thermophilic campylobacters can survive for days (5, 18), weeks (19), and months (8, 30, 37) under various conditions, in a variety of aqueous media. In untreated lake water, survival of *C. jejuni* has been shown to be reduced compared to filter-sterilized lake water (18, 19).

Skelly and Weinstein (31) propose an ecoenvironmental approach to human campylobacteriosis, which acknowledges "the complexity of campylobacter survival trajectories in terms of environmental constraints and ecological filters." They suggest that the ingestion of campylobacters by planktonic grazers in aquatic environments influences their survival and contributes to the complexity of modeling their survival. In support of this idea, it is well established that the freshwater crustacean, *Daphnia*, is a highly efficient grazer of aquatic bacteria (13, 14, 15, 21), having the ability to filter organisms as small as 0.4 to 2 μm (3, 38). Grazing by *Daphnia* has been shown to affect the biomass, productivity, size structure and species composition of aquatic bacterial communities (13). *Daphnia* will also effectively graze *Escherichia coli* (26), however, the ability of *Daphnia* to control concentrations of human pathogenic bacteria, such as *C. jejuni*, has never been tested to our knowledge.

The overall aim of our study was to determine the rate at which *Daphnia carinata* King could remove *C. jejuni* from aqueous media. We used a model experimental system to examine *Daphnia-Campylobacter* interactions and determined grazing and clearance rates of *D. carinata* on *C. jejuni* under simulated natural conditions and in the presence of an algal food source. We also assessed the effect of gut passage on the survival of *C. jejuni* by determining the culturability of *C. jejuni* in the fecal material of *D. carinata* fed on *C. jejuni*.

MATERIALS AND METHODS

Experimental organisms. *Campylobacter jejuni* subsp. *jejuni* strain 825/79, biotype 2, was obtained from the New Zealand Reference Culture Collection (ESR, Wellington, New Zealand) and maintained at 4°C on Columbia sheep blood agar (Fort Richard). The liquid medium used was Preston broth which was comprised of nutrient broth no. 2 (CM 67, Oxoid, Global Scientific), 5% defibrinated horse blood, and Campylobacter Growth Supplement (SR84, Oxoid), but not the Campylobacter Selective Supplement (SR 117, Oxoid) or cefoperazone. Single colonies from Columbia agar plates were placed into 12-ml tubes filled with Preston broth and incubated at 37°C for 24 h. The resulting growth was pelleted by centrifugation (10,000 rpm, 10 min, 4°C), and the cell pellet was washed twice and rediluted in 12 ml of 0.1% peptone (Difco, Fort Richard). This procedure

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consistently provided a concentration of approximately 10^9 CFU ml^{-1} in the final suspension.

Daphnia carinata King was collected from the lower pond at the Dunedin Botanical Gardens, Dunedin, New Zealand, and cultured in COMBO culture medium for freshwater algae and zooplankton (17) over a 3-month period. The *D. carinata* cultures were fed exclusively on a culture of the alga *Cryptomonas* sp. which was grown at 20°C in modified MBL culture medium (33). Adult *D. carinata* used in the experiments generally ranged from 2.3 to 3.2 mm in length, from the top of the head to the base of the tail spine.

Impact of *D. carinata* grazing on *C. jejuni* survival. To determine survival of *C. jejuni* in the presence or absence of *D. carinata*, and to estimate *D. carinata* grazing rates, six sterile glass culture vessels were filled with 250 ml of autoclaved, filtered COMBO culture medium. One ml of *C. jejuni* suspension (10^9 CFU ml^{-1}) and *Cryptomonas* sp. (final concentration, 180 ml^{-1}) was added to all vessels. Then, ten *D. carinata*, which had been rinsed twice in sterile COMBO, were added to three of the six culture vessels. After gentle shaking to mix the contents, the vessels were not agitated during the experiment, which took place in dim light at 13°C, so that *Campylobacter* survived for the duration of the experiment.

At 0, 24, 48, and 72 h, 1-ml samples were taken from the upper third of each culture vessel (to avoid sampling daphnid fecal material) and diluted 10-fold in 0.1% peptone to yield concentrations of *C. jejuni* appropriate for using a most probable number method, described as follows. One ml of the diluted samples was added to tubes (12 ml) containing Preston broth, ensuring that there was minimal headspace. The tubes were incubated at 37°C for 4 to 6 h before the addition of selective supplement (SR 117, Oxoid) and cefoperazone (1.25 mg $liter^{-1}$). Tubes were transferred to 42°C and incubated for a further 24 h. An aliquot from each tube was subsequently streaked onto modified charcoal cefoperazone desoxycholate agar (CCDA; Oxoid, Basingstoke, UK) plates, which were incubated at 42°C in a microaerophilic atmosphere. Confirmation of the presence of *C. jejuni* was based on the examination of colonial morphology, microscopic examination of Gram-stained samples and the results of oxidase testing. Most probable number methods have been shown to enhance recovery of *C. jejuni* from water, compared to drop plate methods (7, 25; P. Bremer, unpublished data).

After sampling at 72 h, the *D. carinata* were removed from the culture vessels, rinsed thoroughly with sterile water, placed in a sterile petri dish and photographed to determine their size. They were then transferred aseptically to vials containing 0.1% peptone (10 ml) and three glass beads (3.5 to 4.5 mm in diameter, BDH) and vortexed for 1 min. The number of *C. jejuni* in the resulting suspensions was estimated using the most probable number method.

To test whether subsampling the medium was an effective method for estimating total culturable *C. jejuni* in the vessels, the medium remaining at the end of the experiment, including any fecal material, was filtered through 0.45-μm filters (catalog no. 66278, Pall Gelman Laboratory) and the concentration of culturable *C. jejuni* on the filter was determined. In addition, to account for potential bacterial adhesion to the vessels, their interior walls were thoroughly wiped with a cotton bud to remove attached bacteria. The filters and cotton buds were placed into 10 ml of 0.1% peptone. The total number of culturable *C. jejuni* from each vessel was converted to a concentration per ml of medium to compare with the bacterial concentration in subsamples of the medium at 72 h, immediately before filtration.

***C. jejuni* survival following ingestion and excretion by *D. carinata*.** Three autoclaved glass culture vessels were filled with 250 ml of autoclaved, filtered COMBO medium. Ten *D. carinata*, which had been rinsed twice in sterile COMBO, and 1 ml of *C. jejuni* suspension were added to each vessel. After initial gentle shaking, the vessels were incubated at 13°C in dim light.

At the start of the experiment, 1-ml subsamples were taken from the upper third of the culture vessels to determine the starting concentration of *C. jejuni* by the most probable number method. After 2 h, additional subsamples were taken to determine the concentration of *C. jejuni* in the medium. Subsequently, five of the 10 *D. carinata* in each vessel were collected, rinsed twice with sterile COMBO, and assayed to determine associated *C. jejuni* numbers, using the most probable number method. The remaining five *D. carinata* from each vessel were transferred to fresh, sterile COMBO medium for a further 30 min, before they were removed, rinsed, and assayed to determine *C. jejuni* numbers. The remaining COMBO, including any fecal material, was filtered and the concentration of *C. jejuni* determined using the most probable number method.

Clearance rates. Clearance rates were calculated from the log-linear slopes of relationships between bacterial concentration and time, estimated by least squares linear regression. It was assumed that grazing rates were independent of bacterial densities. The proportion of the population grazed per unit time (g) was calculated as $g = 1–10^{(m1-m2)}$, where $m1$ and $m2$ are the log-linear regression slopes of bacterial concentration versus time for *Daphnia* treatments and controls, respectively. Clearance rates (F, ml $animal^{-1}$ h^{-1}) were calculated as $F = vgn^{-1}$ where v is the volume (ml) of the culture and n is the number of animals in the culture.

This method yielded similar but more accurate estimates of clearance rates than the formulae in Marin et al. (24), which used only initial and final bacterial concentrations.

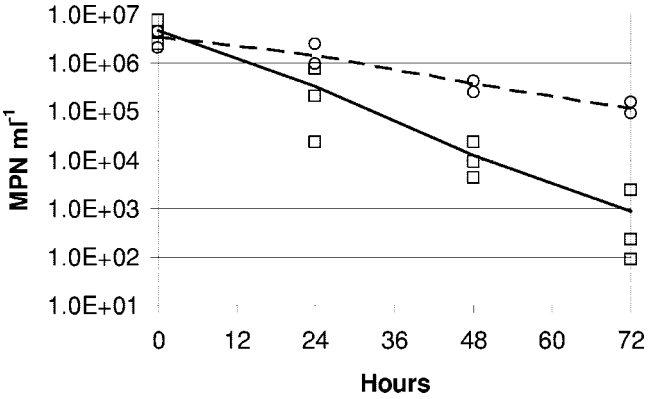

FIG. 1. *Daphnia carinata* grazing on *Campylobacter jejuni* as determined by a most probable number method. Points are treatment replicates showing *C. jejuni* concentrations in COMBO in the presence (squares, solid line) and absence (circles, dashed line) of *D. carinata*.

RESULTS

Impact of *D. carinata* grazing on *C. jejuni* survival. The rate of decline of *C. jejuni* concentration during the grazing experiment was significantly higher in the presence of *D. carinata* than in their absence (Fig. 1). After 72 h, *D. carinata* had reduced *C. jejuni* survival by ca. 2 orders of magnitude. A grazing rate (corrected for nongrazing mortality in the *Daphnia*-free culture vessels) of 7% of *C. jejuni* h^{-1} by the 10 *D. carinata* was calculated at a daphnid density of 40 $liter^{-1}$, a *C. jejuni* density of between 1.4×10^6 and 1.0×10^3 most probable number ml^{-1}, at 13°C, in the presence of *Cryptomonas* sp. This grazing rate corresponded to a clearance rate of 1.75 ml ind^{-1} h^{-1} ($R^2 = 0.998$, $P < 0.001$).

Comparison of the treatments with and without *D. carinata* revealed that *D. carinata* effectively rendered the *C. jejuni* nonculturable (Fig. 2). The bacterial concentration recovered from the filtered medium was not significantly different to that recovered from the unfiltered medium (Fig. 2).

Assays for *C. jejuni* on or in the *D. carinata* at the end of the experiment revealed an average of 33 cells of *C. jejuni* associated with each animal.

***C. jejuni* survival following ingestion by *D. carinata*.** In experiments where *D. carinata* were placed in culture vessels containing *C. jejuni* at 1.3×10^9 cells $vessel^{-1}$, an average of 2.3×10^8 cells $vessel^{-1}$ (or 18% of the cells at $t = 0$) remained in the medium after 2 h. At this time, the *D. carinata* in each vessel accounted for 1.2×10^7 *C. jejuni*, either attached to the *Daphnia* or in their digestive tracts. After *D. carinata* were placed in sterile medium for 30 min, the number of bacteria associated with them was 3.2×10^5 cells $vessel^{-1}$ with a further 7.9×10^5 cells $vessel^{-1}$ recovered from the total volume of culture medium including fecal material. Therefore, during the 30 min in sterile medium, bacterial numbers initially associated

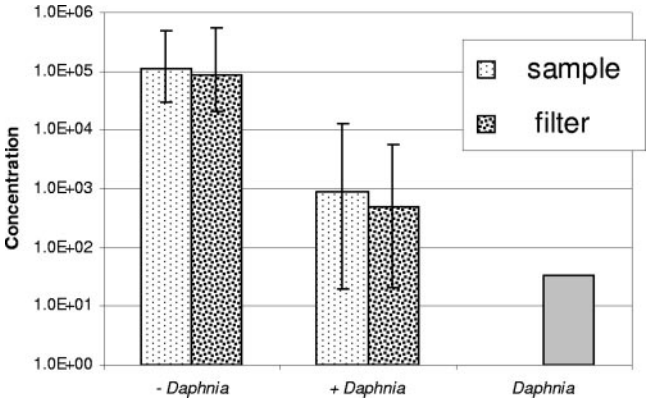

FIG. 2. Comparison of counts of *Campylobacter jejuni* in experimental culture vessels after 72 h, as determined by subsampling COMBO medium and by filtering the entire contents of culture vessels (with *Daphnia* and without *Daphnia*; most probable number ml^{-1}). Vertical lines indicate the maximum and minimum of three replicates. The count of *C. jejuni* associated with *Daphnia carinata* in one treatment replicate after 72 h (*Daphnia*; most probable number $animal^{-1}$).

with the transferred *D. carinata* declined by 91%, indicating that the bacteria were killed by association with *D. carinata*, presumably during passage through the gut.

DISCUSSION

Previous studies of *C. jejuni* in aqueous media have shown that the period of survival in water is generally inversely proportional to temperature of the medium, within the range of ca. 4 to 40°C (5, 18, 19, 27, 30, 36, 37). Different strains, isolated from different sources, show considerable variability in survival parameters (8). Other environmental factors such as UVB light level, the level of oxygenation, water source and the presence of other microorganisms have been reported to influence *C. jejuni* survival (5, 7, 27, 36). Korhonen and Martikainen (18, 19) showed that survival of *C. jejuni* was significantly higher in filter-sterilised (0.2-μm-filtered) lake water than in untreated lake water or 5.0-μm-filtered lake water. The authors suggested that predation by protozoans may have reduced survival of *C. jejuni* in unsterilised lake waters, as has been shown to influence *E. coli* survival in previous studies (references 18 and 19 and references therein). Indeed, once in the aquatic environment, *C. jejuni* becomes a component of the aquatic microbial food web and its survival could potentially be affected by predation.

We used a model system for studying the interactions between *D. carinata* and *C. jejuni* in the presence of a *Cryptomonas* sp. as a supplementary algal food source for *Daphnia*. We found that the presence of *D. carinata* strongly reduced *C. jejuni* numbers in COMBO culture medium (Fig. 1 and 2). Furthermore, it appears that the association of *C. jejuni* with *D. carinata* results in death of the *C. jejuni* rather than sequestration. We did not determine the nature of this association but suggest that the death of *C. jejuni* cells was most likely due to their ingestion by *D. carinata*.

Daphnia are effective grazers of bacteria in lakes and ponds, with typical bacterial clearance rates ranging from 0.1 to 2.8 ml ind^{-1} h^{-1} (13, 29, 38). We have demonstrated that *D. carinata* can clear water of *C. jejuni* under experimental conditions at a rate of 1.75 ml ind^{-1} h^{-1}. Given that *C. jejuni* cannot reproduce in waterways and is typically larger than most of the bacteria present in surface waters (0.1 to 1 μm) (11, 13), it is likely that the grazing activity of *D. carinata* and other daphnids could reduce the abundance of *C. jejuni* more effectively than that of native aquatic bacteria in aquatic ecosystems.

The calculated clearance rate of *C. jejuni* of 1.75 ml ind^{-1} h^{-1} falls within the range of clearance rates of *Daphnia* grazing on aquatic bacterial assemblages (13) and is similar to that for *D. carinata* grazing on *E. coli* at 18°C in a preliminary experiment (P. Bremer, unpublished data). *Daphnia* commonly occur at densities >30 ind $liter^{-1}$ and can exceed 100 ind $liter^{-1}$ (4). At a clearance rate of 1.75 ml ind^{-1} h^{-1}, *Daphnia* at a density of 25 ind $liter^{-1}$ could theoretically clear a water body of nondividing *Campylobacter* cells in <24 h. Our clearance rate is calculated for a range of *Campylobacter* concentrations that is typical of wastewaters (12, 31); therefore, further study is required to confirm similar clearance rates at lower concentrations of *C. jejuni*, more typical of surface waters (9, 31).

Grazing by the protozoan *Cyclidium glaucoma* has recently been shown to reduce the survival of another pathogenic bacterium, *Vibrio cholerae*, in brackish waters (28). Our study appears to be the first to show that crustacean zooplankton can reduce the concentration of human pathogens under simulated natural conditions. We confirmed that our model system could be used to explore the role of microbial food webs in regulating bacterial pathogen abundance in aquatic ecosystems. The results of our study suggest that the use of food web biomanipulation in recreational water bodies and drinking water reservoirs could enhance *Daphnia* densities and reduce pathogen density and the risk of contracting waterborne diseases.

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