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Survival of *Campylobacter jejuni* in Waterborne Protozoa

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The failure to reduce the *Campylobacter* contamination of intensively reared poultry may be partially due to *Campylobacter* resisting disinfection in water after their internalization by waterborne protozoa. *Campylobacter jejuni* and a variety of waterborne protozoa, including ciliates, flagellates, and alveolates, were detected in the drinking water of intensively reared poultry by a combination of culture and molecular techniques. An in vitro assay showed that *C. jejuni* remained viable when internalized by *Tetrahymena pyriformis* and *Acanthamoeba castellanii* for significantly longer (up to 36 h) than when they were in purely a planktonic state. The internalized *Campylobacter* were also significantly more resistant to disinfection than planktonic organisms. Collectively, our results strongly suggest that protozoa in broiler drinking water systems can delay the decline of *Campylobacter* viability and increase *Campylobacter* disinfection resistance, thus increasing the potential of *Campylobacter* to colonize broilers.

Campylobacter jejuni is recognized as one of the leading causes of food-borne disease in the developed world (2, 4, 39). Estimates suggest that in the United Kingdom and the United States 1.1 and 1%, respectively, of the populations are affected annually by *Campylobacter* infection, and substantial sums are lost due to clinical costs and lost working hours, e.g., between $1.3 and $6.2 billion in the United States alone (26, 35, 50). Campylobacteriosis usually involves a self-limited gastrointestinal illness lasting up to 1 week, after an incubation period of 1 to 7 days (20, 22, 58). More than 90% of campylobacteriosis cases are sporadic, with the consumption of (and cross-contamination from) undercooked poultry an identified risk factor (4, 12, 28, 32). An extensive Food Safety Authority survey, conducted between April and June 2001, found the United Kingdom national average of *Campylobacter* contamination to be 50% in finished raw chicken meat (including both whole and portioned samples) (24).

The management of infection in breeder flocks appears to have relatively little importance in the epidemiology of infection since most researchers have found no compelling evidence that campylobacters are transmitted vertically (12, 14, 21, 65, 67). It is most likely that chicks become colonized from environmental sources, e.g., unchlorinated drinking water (22). Intensively reared broiler chickens readily pick up *C. jejuni* from the environment and, since campylobacters have a wide range of hosts, there are many potential sources of infection (4, 21, 57). In the developed world, a variety of biosecurity measures, e.g., boot dips and hygiene barriers, are generally practiced on broiler farms (27, 56). However, despite these measures, broilers still have high levels of *Campylobacter* contamination, e.g., 10^5 to 10^9 CFU per g of intestinal contents (12, 48, 66). The presence of *Campylobacter* in the intestinal tract implicates ingestion of a contaminated source (45). Neither feeds nor fresh litter seem to be likely sources of *Campylobacter*. Commercial feeds are dried, are pelleted, contain little moisture (8 to 10%), tend to be pasteurized, and are air blown into silos (45, 57). The litter used on farms is generally wood shavings which are dry and resinous (being mainly softwood) and normally come directly from sawmills (57).

Drinking water has sometimes been found to be a significant source of infection (12). Viable, nonculturable organisms in water may be important in *C. jejuni* transmission, but efforts to infect day-old hatched chicks have been variable, and the significance of this source is still under review. In addition to free suspensions, bacteria in water systems also exist attached to sediment or in biofilms on submerged surfaces, where these communities usually consist of bacteria, fungi, algae, and protozoa with high grazing activity (73). Aquatic biofilms may harbor potential human pathogens, e.g., *C. jejuni*, and promote their survival through a variety of mechanisms such as uptake by protozoa, resulting in protection from disinfection (73). King et al. (37) demonstrated that *C. jejuni*, when ingested by the protozoan *Tetrahymena pyriformis*, was more than 50 times more resistant to free chlorine (1 mg per liter, pH 7.0 at 25°C) than freely suspended *C. jejuni* (37). The potential and significance of protozoa to act as transfer vehicles for *Campylobacter* to infect intensively reared poultry is unclear. This is because there is currently very little information on the identity of eukaryotic microbes in poultry drinking water systems and of possible interactions between protozoa and *Campylobacter*. We describe here a combination of culture-based and molecular techniques to assess the potential of waterborne protozoa to act as vehicles for the *Campylobacter* infection of intensively reared poultry.

MATERIALS AND METHODS

Broiler house biosecurity. All broiler houses contained between 10,000 and 20,000 cock Cob breed 500 broilers. One-day-old chicks were delivered to the farms and placed on litter consisting of wood shavings coming directly from sawmills. Pasteurized feed was supplied by an automatic auger system with feed pans, and water was provided through nipple drinker systems. Between each

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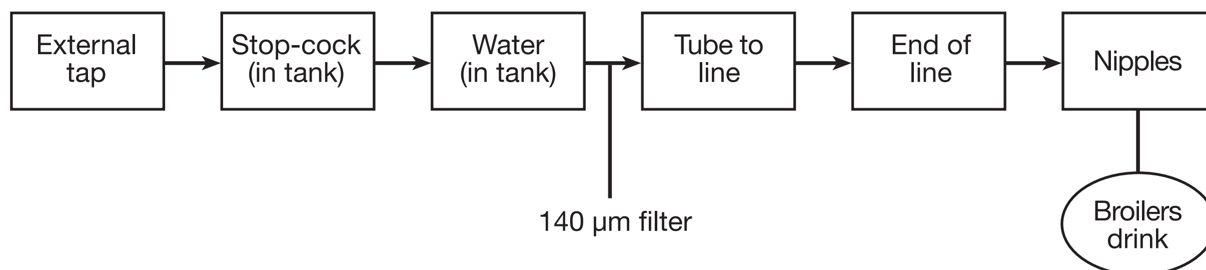

FIG. 1. Flow diagram of drinking water systems in the broiler houses of intensively reared poultry. The broilers drink the water, obtained from main water supplies, at the nipples.

flock, litter was removed from houses, which were then cleaned and disinfected. Broilers fed and drank when their houses were lit for 24 h when they were 1 day old, and when broilers were 7 days old, lighting was reduced to 4 h per day. All of the houses sampled in this investigation had a demarcation zone between the outside and inside of the house "marked" by a stepover bench. Each broiler house had separate overalls and Wellington boots (inside and outside). A disinfectant boot dip with a 1:20 dilution of Virudine (DuPont, Sudbury, Suffolk, United Kingdom), an iodophor disinfectant with 2.8% iodine and 28% phosphoric acid activity, which was supposed to be replenished weekly, were present outside each broiler house. All of the water systems in houses were supposed to be disinfected by farmers between every two flocks of broilers using a 1:600 dilution of Virudine, where the diluted disinfectant was pumped though the water system, left overnight, and then removed. Farmers performed daily checks of the houses, recording a variety of information, e.g., age, broiler weight, cumulative mortality, feed delivery, water meter readings, and house temperature. Members of the poultry industry performed audits before the broilers arrived and when they were 4 weeks old, where the presence of biosecurity measures, e.g., the presence of a hygiene barrier, was assessed and recorded.

Microbiological analysis. Feces/bedding and cloacal swab samples were taken from a single broiler house at five different farms. Fifteen cloacal swabs were taken from each broiler house, immediately placed into 5 ml of Preston broth (Oxoid, Basingstoke, Hampshire, United Kingdom), and transported to the laboratory at 4°C, where they were microaerophilically incubated for 48 h at 37°C (63). Each set of enrichment broths was pooled and filtered (0.6-μm pore size; Whatman, Middlesex, United Kingdom), and the flowthrough was centrifuged (9,300 × *g* for 5 min) (63). Most of the supernatant was discarded, the pellet was briefly vortexed in the remaining 1 ml of supernatant, and 0.1 ml of the suspension was incubated microaerophilically using CampyGen gas packs (Oxoid) in 3.5-liter gas jars (Oxoid) for 24 h at 42°C on Preston agar plates (Oxoid). Individual colonies were streaked for purity onto Preston agar plates and grown microaerophilically for 24 h at 42°C. Gram staining and biochemical testing using Mast ID Camp Identification Systems (Mast Diagnostics, Bootle, United Kingdom) were performed to check for vibroid morphology and for hippurate hydrolysis and indoxyl acetate and urease activity, respectively.

Water analysis. Four of the five farms in this investigation used water directly from mains supply, and the remaining farm used a bore-hole source. Where possible, 510-ml water samples were taken from six places in the broiler drinking water systems (Fig. 1). Nipples were cleaned with 70% alcohol before water collection. An aliquot of water sample (10 ml) was screened microscopically for the presence of protozoa. Water samples were filtered by using nitrocellulose membranes (0.2-μm pore size; Whatman). DNA was then extracted from the filters by using DNA SPIN Kits for Soil (Bio 101, Anachem, Bedfordshire, United Kingdom) and processed according to the manufacturer's instructions.

PCR detection of *Campylobacter* and protozoa. All DNA extractions were performed using DNA SPIN Kits for Soil and processed according to the manufacturer's instructions. Bovine serum albumin (BSA) was obtained from Ambion, Cambridgeshire, United Kingdom. Unless stated otherwise, all other components used to perform PCR amplifications, purifications, and cloning were obtained from Invitrogen, Paisley, United Kingdom. DNA extractions were performed on *Campylobacter* colonies from the Preston agar plates. DNA was also extracted from samples of feces and bedding material and from water filtrates. Approximately 1 g of feces/bedding was taken from each of five locations randomly chosen within each broiler house. These were mixed in a sterile container and then placed on ice. DNA extractions were performed on 500 mg of each of these feces/bedding samples. The presence of *C. jejuni* and protozoa from environmental systems were determined by using the Winters et al. (78) seminested PCR system and the Einsele et al. (18) eukaryotic rRNA PCR system, respectively (Table 1). The seminested PCR amplifications for *C. jejuni* were performed in volumes of 100 μl containing 2.0 μl of DNA extracts, 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase, 1.0 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 500 ng of BSA μl^{-1}, and 200 μM concentrations of each deoxynucleoside triphosphate (78). For the first round of PCR, together with the primers WIN1 and WIN2 (Table 1), a protocol consisting of 1 cycle of 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, with a final 3 min at 72°C was used (78). PCR product from the first round was used as the DNA template for the second round, together with primers WIN1 and WIN3 (Table 1). Twenty-four cycles were used in the second round with the conditions being the same, except for a lower annealing temperature (53°C) (78). For each of the three *C. jejuni* isolates a 122-bp amplicon from the Cj0343c gene was generated by using the Winters et al. (78) seminested PCR system (Table 2).

Amplification reactions for the detection of eukaryotic rRNA were performed in final volumes of 100 μl containing 2.0 μl of DNA, 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase, 3 mM $MgCl_2$, 51 mM KCl, 20 mM Tris-HCl (pH 8.4), 200 μM concentrations of each dNTP, and 500 ng of BSA μl^{-1}. Using EIN1 and EIN2 primers (Table 1), a PCR protocol consisting of 1 cycle of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, with a final 5 min at 72°C was used (18). The technique of "band-stab PCR" was used to obtain a single desired PCR product of 500 bp (8). All PCR product purifications were performed by using Concert Rapid PCR purification kits, and the products were examined by using agarose gel electrophoresis. Each gel was composed of 1.5% multipurpose agarose (Boehringer, Ingelheim, Germany), stained with 0.5 μg of ethidium bromide ml^{-1}, subjected to 100 V for 90 min, and scanned with a White/UV Transilluminator UVP Transilluminator (Ultra-Violet Products, Cambridge, United Kingdom).

Sequencing PCR products. All PCR products (detected from water and feces/bedding samples) for DNA sequencing were first cloned using Original TA cloning kits. Samples were prepared for sequencing using a BigDye Terminator V2.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) ethanol precipitation as described in the kit protocol and an ABI/Hitachi 3100 Genetic Analyzer capillary action sequencer (ABI/Hitachi, Arcade, NY). The forward

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′-3′)	Annealing position	Reference
WIN1	AAA TAA AGT TAG AGG TAG AAT GT	*C. jejuni* NCTC 11168, coordinates 66984 to 67006 in genome; Cj0343c gene	78
WIN2	GGA TAA GCA CTA GCT AGC TGA T	*C. jejuni* NCTC 11168, coordinates 67140 to 67121 in genome; Cj0343c gene	78
WIN3	GCA CGC CTA AAC CTA TAG C	*C. jejuni* NCTC 11168, coordinates 67105 to 67087 in genome; Cj0343c gene	78
EIN1	ATT GGA GGG CAA GTC TGG TG	*T. pyriformis*, coordinates 537 to 557 in small subunit of rRNA	18
EIN2	CCG ATC CCT AGT CGG AT AG	*T. pyriformis*, coordinates 998 to 1024 in small subunit of rRNA	18

TABLE 2. Library of *C. jejuni* clones with sample collection data

Clone name	Sample data	GenBank no.
1A	Farm 1, feces/bedding	AY830861
1B	Farm 1, feces/bedding	AY830862
2A	Farm 1, tank water	AY830863
2B	Farm 1, tank water	AY830864
3A	Farm 4, feces/bedding	AY830865
3B	Farm 4, feces/bedding	AY830866
4A	Farm 4, end of line	AY830867
4B	Farm 4, end of line	AY830868
5A	Farm 4, tube to line	AY830869
5B	Farm 4, tube to line	AY830870
6A	Farm 4, tank water	AY830871
6B	Farm 4, tank water	AY830872
7A	Farm 5, feces/bedding	AY830873
7B	Farm 5, feces/bedding	AY830874
8A	Farm 5, end of line	AY830875
8B	Farm 5, end of line	AY830876
9A	Farm 5, tube to line	AY830877
9B	Farm 5, tube to line	AY830878
10A	Farm 5, stop-cock water (in tank)	AY830879
10B	Farm 5, stop-cock water (in tank)	AY830880
11S	Farm 1, cloacal swab	AY830881
12S	Farm 4, cloacal swab	AY830882
13S	Farm 5, cloacal swab	AY830883

strands of the three isolated *C. jejuni* strains and two clones from each of the 10 *C. jejuni* environmental amplicons were sequenced; these sequences were then deposited at the GenBank database under accession numbers AY830861 to AY830883 (Table 2). Forward and reverse DNA strands were also sequenced from 34 random eukaryotic clones—3 from each external tap and tank sample and 2 from each nipple water sample—and were then deposited at the GenBank database under accession numbers AY837467 to AY837500 (Table 3). Sequences were then analyzed by using Chromas version 1.62 (Technelysium, Tewantin Qld, Australia), NCBI BLAST, and EMBL-EBI (European Bioinformatics Institute, Heidelberg, Germany) CLUSTAL W alignment. A phylogenetic tree of *Campylobacter* amplicons was constructed by using the neighbor-joining method based on all nucleotide sites, with corrections for multiple substitutions by the Jukes Cantor method in MEGA version 2.1 (The Pennsylvania State University, University Park, PA).

Internalization of *Campylobacter* by protozoa. All of the following microscopy coculture assays were performed in triplicate, and the *Campylobacter* viability decline and disinfection assays were each performed in quadruplicate. *T. pyriformis* (CCAP 1630/14A) (Culture Collection of Algae and Protozoa, Oban, United Kingdom) and *Acanthamoeba castellanii* (CCAP 1501/10) were used in all in vitro experiments measuring bacterial-protozoan interactions. Protozoa were enumerated by using direct hemocytometer counts. A drop of 37% formaldehyde (Sigma, St. Louis, MO) was added to 1-ml aliquots of suspensions of *T. pyriformis* to suppress motility and to make the cells easier to count. Fluorescence microscopy used a Super High-Pressure Nikon Mercury Lamp (Kawasaki, Kanagawa, Japan) and B-2A and/or UV-1A Nikon filters, and images were recorded by using Kroma Scan (Kinetic Imaging, 2000, Nottingham, United Kingdom).

Establishing cocultures. During this research, protozoa were grown for 3 days, starved, and then placed in low-nutrient conditions to replicate the low-nutrient conditions in water systems, minimize protozoa variation in feeding behavior, and increase protozoan ingestion of *Campylobacter* (29, 30, 68). *A. castellanii* organisms were grown for 3 days in 20 ml of proteose peptone glucose broth (CCAP) at 25°C to a population density of 10^6 cells ml^{-1}. *T. pyriformis* organisms were grown for 3 days in 15 ml of proteose peptone yeast extract broth (CCAP) at 25°C to a population density of 7.5×10^5 cells ml^{-1}. Cultures were gravity filtered by using membrane filters (0.8-μm pore size; Whatman) to remove broth. Cells were then resuspended in a 1:1 dilution of the appropriate culture broth and Page's ameba saline (PAS) solution (CCAP; total volume, 10 ml) and incubated at 25°C for 12 h. This was done to reduce the effects of osmotic shock to protozoa and to avoid cyst formation (16, 37). After 12 h, the cells were filtered again, as described above, resuspended in 5 ml of PAS solution, adjusted to a concentration of 10^8 cells ml^{-1}, and incubated at 25°C for 12 h. Campylobacters were grown on Preston agar in microaerophilic conditions for 24 h at 42°C using CampyGen (Oxoid) gas packs in 3.5-liter anaerobic jars (Oxoid). Bacterial cells were resuspended in PAS, and optical densities were measured at 600 nm.

Determination of viability of internalized bacteria cells. Live/Dead Baclight bacterial viability kits (Invitrogen) utilize SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain propidium iodide. Bacteria with intact membranes fluoresced green (alive), whereas bacteria with damaged membranes fluoresced red (dead). *A. castellanii* and *T. pyriformis* were prepared for coculture as previously described. *C. jejuni* NCTC 11351 (National Collection of Type Cultures), *C. coli* NCTC 11366 and *C. jejuni* subsp. *jejuni* (poultry isolate) were each grown for 24 h, suspended in 10 ml of phosphate-buffered saline (PBS; Oxoid), and adjusted to concentrations of 2.8×10^8 CFU ml^{-1} (optical density at 600 nm of 0.4). Each *Campylobacter* suspension was then stained by using Baclight according to the kit protocol. The *Campylobacter* suspensions were vortexed briefly in 10 ml of PBS and centrifuged for 1 min at 9,300 × *g*; the supernatant was then discarded. Each dyed bacterial pellet was then resuspended in 10 ml of PAS solution.

Cocultures of each protozoa and *Campylobacter* were obtained by adding by 2 ml of protozoan suspension and 0.36 ml of bacterial suspension to 17.64 ml of PAS solution, giving 1:1 ratios of protozoa to *Campylobacter*. The cocultures were incubated at 25°C for up to 24 h and were monitored at time intervals (1 h, 3 h, 6 h, 24 h, and 3 days) over this period. After the appropriate time period, 3 ml of coculture was gravity filtered, and each filter was carefully rinsed with 25 ml (5 by 5 ml) of PAS solution to remove *Campylobacter* from the surface of the protozoa. The invaded protozoa were then observed by using phase-contrast microscopy and fluorescence microscopy (×40 and ×100 Nikon lenses) with B-2A and U-1A filters (Nikon).

Distinguishing internal from external *Campylobacter* from a coculture. *A. castellanii* and *T. pyriformis* were prepared for coculture with *Campylobacter*, as previously described. *C. jejuni* NCTC 11351, *C. jejuni* subsp. *jejuni* (poultry isolate), and *C. coli* NCTC 11366 were each grown for 24 h, suspended in 10 ml of PBS, and adjusted to a concentration of 2.8×10^8 CFU ml^{-1}. Each *Campylobacter* suspension was centrifuged at 9,300 × *g* for 1 min, and the supernatant was removed. Bacterial pellets were stained with 50 μl of fluorescein-isothiocyanate (FITC)-labeled rabbit antibody to *C. jejuni* (AMS Biotechnology, Ltd., Abingdon, United Kingdom), vortexed briefly, and left in darkness for 15 min on ice. Each sample was centrifuged as described above, and the supernatant was removed. The stained bacterial pellets were rinsed in 10 ml of PBS, and the supernatant was discarded. The stained bacterial pellets (protected from light) were then resuspended in 10 ml of PAS solution and vortexed briefly. Cocultures of protozoa and *Campylobacter* were obtained by adding by adding 2 ml of protozoan suspension and 0.36 ml of bacterial suspension to 17.64 ml of PAS, giving 1:1 ratios of protozoa to *Campylobacter*. The cocultures were incubated at 25°C for up to 24 h and were monitored at time intervals (1 h, 3 h, 6 h, and 24 h) over this period. After the appropriate time period, 1 ml of coculture was removed, and 0.01 mg of DAPI (4′,6′-diamidino-2-phenylindole; Sigma) was added to the aliquot, which was then left to incubate at room temperature for 10 min in darkness. The invaded protozoa were then observed by using fluorescence microscopy using ×40 and ×100 phase-contrast lenses (Nikon) and B-2A (FITC) and UV-1A (DAPI) Nikon filters. As negative controls, 50 μl of FITC antibody was added to separate 20-ml suspensions of *A. castellanii* and *T. pyriformis* (10^7 cells ml^{-1}) in PAS in the absence of bacteria. The negative protozoan controls fluoresced after staining with DAPI.

The survival of *Campylobacter* in cocultures. Sonication (on ice) with a microtip probe for 10 s at 40 W appeared to have no effect on the viability of *Campylobacter*, and phase-contrast microscopy clearly confirmed that this treatment completely ruptured *A. castellanii* and *T. pyriformis* cells (37). *A. castellanii* and *T. pyriformis* were prepared for coculture with *C. jejuni* NCTC 11351, *C. jejuni* subsp. *jejuni* (poultry isolate), and *C. coli* NCTC 11366 as previously described. Each coculture was then incubated at 25°C, and at various time intervals (0 h, 3 h, 6 h, and then daily) 1 ml of coculture was removed and sonicated (on ice) for 10 s at 40 W; viable counts (24 h at 42°C on Preston agar) were then performed daily until viable cells were no longer obtained (37). As controls, each *Campylobacter* strain was grown for 24 h at 42°C, suspended in 10 ml of PAS solution, and adjusted to concentrations of 2.8×10^8 CFU ml^{-1}. Concentrations of 10^7 *Campylobacter* CFU ml^{-1} were obtained by adding by 0.36 ml of the bacterial suspension to 19.64 ml of PAS solution. The bacterial suspensions were then incubated at 25°C. At various time intervals (0 h, 3 h, 6 h, and then daily), 1 ml of planktonic suspension was removed for each *Campylobacter* strain, and viable counts were then performed daily until viable cells were no longer obtained.

***Campylobacter* disinfection resistance studies.** Disinfection assays were mainly based on a previous resistance study by King et al. (37). A variety of preliminary

TABLE 3. Library of eukaryotic clones with sample collection data

Clone	Sample data	GenBank no.	Most closely related microbe, % similarity
1A	Farm 1, external tap	AY837467	*Trichosporon montevideense*, 99% small subunit rRNA
1B	Farm 1, external tap	AY837468	*Trichosporon montevideense*, 99% small subunit rRNA
1C	Farm 1, external tap	AY837469	*Trichosporon montevideense*, 97% small subunit rRNA
2A	Farm 4, external tap	AY837470	*Phialocephala fortinii*, 91% small subunit rRNA
2B	Farm 4, external tap	AY837471	*Botryotinia fuckeliana*, 18S 99% rRNA
2C	Farm 4, external tap	AY837472	*Botryotinia fuckeliana*, 99% 18S rRNA
3A	Farm 5, external tap	AY837473	*Scopulariopsis brevicaulis*, 99% 18S rRNA
3B	Farm 5, external tap	AY837474	*Paecilomyces lilacinus*, 98% 18S rRNA
3C	Farm 5, external tap	AY837475	*Debaryomyces hansenii* var. *fabryi*, 98% 18S rRNA
4A	Farm 1, tank	AY837476	*Paraurostyla weissei* 17S, 90% rRNA gene,
4B	Farm 1, tank	AY837477	Uncultured alveolate clone LEMD251, 93% small subunit rRNA
4C	Farm 1, tank	AY837478	*Trichosporon montevideense*, 99% small subunit rRNA
5A	Farm 2, tank	AY837479	*Paraphysomonas butcheri*, 96% 18S rRNA
5B	Farm 2, tank	AY837480	*Eurotium herbariorum*, 98% 18S rRNA gene
5C	Farm 2, tank	AY837481	*Eurotium herbariorum*, 97% 18S rRNA gene
6A	Farm 3, tank	AY837482	*Hydnum rufescens* 18S, 96% rRNA gene
6B	Farm 3, tank	AY837483	*Spumella oblique*, 96% 18S rDNA gene
6C	Farm 3, tank	AY837484	*Scopulariopsis brevicaulis*, 97% 18S rRNA gene,
7A	Farm 4, tank	AY837485	*Phialophora verrucosa*, 99% 18S rRNA
7B	Farm 4, tank	AY837486	*Phialophora verrucosa*, 98% 18S rRNA
7C	Farm 4, tank	AY837487	Unidentified eukaryote clone LKM67, 97% 18S rRNA
8A	Farm 5, tank	AY837488	*Scopulariopsis brevicaulis*, 97% 18S rRNA gene
8B	Farm 5, tank	AY837489	Uncultured alveolate clone LEMD251, 95% small subunit rRNA
8C	Farm 5, tank	AY837490	Uncultured alveolate clone LEMD251, 95% small subunit rRNA
9A	Farm 1, nipples	AY837491	*Trichosporon montevideense*, 98% small subunit rRNA
9B	Farm 1, nipples	AY837492	*Aspidisca steini*, 92% small subunit rRNA
10A	Farm 2, nipples	AY837493	*Eurotium herbariorum*, 99% 18S rRNA gene
10B	Farm 2, nipples	AY837494	*Gibberella pulicaris*, 99% 18S rRNA
10B	Farm 2, nipples	AY837494	*Gibberella pulicaris*, 99% 18S rRNA
11A	Farm 3, nipples	AY837495	Uncultured alveolate clone LEMD251, 94% small subunit rRNA
12A	Farm 4, nipples	AY837497	*Capronia coronata*, 97% 18S rRNA
12B	Farm 4, nipples	AY837498	*Penicillium italicum*, 94% 18S rRNA
13A	Farm 5, nipples	AY837499	*Cladosporium cladosporioides*, 99% 18S rRNA
13B	Farm 5, nipples	AY837500	*Gibberella pulicaris*, 96% 18S rRNA

experiments were conducted to verify the functionality of the system for analysis of bacterial viability in a protozoan model. First, it was confirmed that after exposure to 10% sodium thiosulfate (STS; Sigma) in PAS, no significant effects ($P > 0.05$) on the growth of *Campylobacter*, *T. pyriformis*, and *A. castellanii* were observed (data not shown). A 1:1,000 dilution of the disinfectant Virudine was found to kill planktonic *Campylobacter* but had no significant effect ($P > 0.05$) on the growth *T. pyriformis* and *A. castellanii* in PAS at 25°C (data not shown). This concentration was used for all subsequent disinfection experiments.

Cocultures of *C. jejuni* NCTC 11351, *C. jejuni* subsp. *jejuni* (poultry isolate), and *C. coli* NCTC 11366 with *T. pyriformis* and *A. castellanii* were each prepared as previously described, and each coculture was incubated for up to 24 h at 25°C. During the 24-h incubation period and after 3, 6, 9, 12, and 24 h, a 1:1,000 dilution of Virudine was used to kill planktonic *C. jejuni*; 2.2 ml of 1:100 Virudine was then added to each coculture for a contact time of 1 min. Then, 2.2 ml of sterile 10% STS (Sigma) was added to neutralize the disinfectant, and each sample was gravity filtered (0.8 μm) and rinsed with 7 ml of PAS. Serial dilutions of the filtrate were then performed to quantify the number of *Campylobacter* on the surface of protozoa that survived disinfection. Next, the filter was resuspended in 10 ml of PAS, 1 ml of coculture was removed and sonicated (on ice) for 10 s at 40 W, and viable *Campylobacter* counts (24 h at 42°C on Preston agar) were performed.

To examine the effect of the age of protozoa on *Campylobacter* disinfection resistance, coculture and disinfection assays were performed as previously described, except that before coculture incubation protozoa were grown 3, 6, and 9 days, and only a coculture time of 3 h was used.

Statistical analysis. To determine whether values were significantly different ($P < 0.05$) between *Campylobacter* strains in a planktonic state compared to when they were in the presence of protozoa (cocultures), strain data were compared by using SPSS 11.0 software (SPSS, Inc., Chicago, IL) using the Bonferroni (one-way analysis of variance) multiple comparison test.

RESULTS

***C. jejuni* detected in poultry broiler drinking water.** Strains of *C. jejuni* were recovered from broiler cloacal swabs, and this organism was also detected by seminested PCR in the drinking water and litter/feces on farms 1, 4, and 5 (Table 2). Identification was based on Gram staining and biochemical tests and confirmed by sequence analysis of seminested PCR products. No *C. jejuni* was detected in farms 2 and 3. In all instances, on farms where *C. jejuni* cultures were recovered from broilers a positive PCR result was also obtained from water analysis of the farm (Fig. 2A). All positive seminested 122-bp PCR *C. jejuni* products recovered from the various water samples were cloned and sequenced to confirm the specificity of the assay. Two clones of each amplicon were selected for sequencing (Table 2). The *C. jejuni* amplicons derived from cloacal swabs were not cloned. The homology studies of *C. jejuni* and sequence alignment data (Fig. 2B and C) illustrate that conserved regions of *C. jejuni* were amplified. In the dendrogram 2A, 2B, and 11S were grouped together, suggesting that the same strain of *C. jejuni* in broilers was also present in tank water from farm 1 (Fig. 2B). The distance between clones 1A and 1B in the dendrogram suggests that more than one *C. jejuni* strain was present in the feces/litter of farm 1. The *C. jejuni* amplicons all showed a high degree of homology to gene

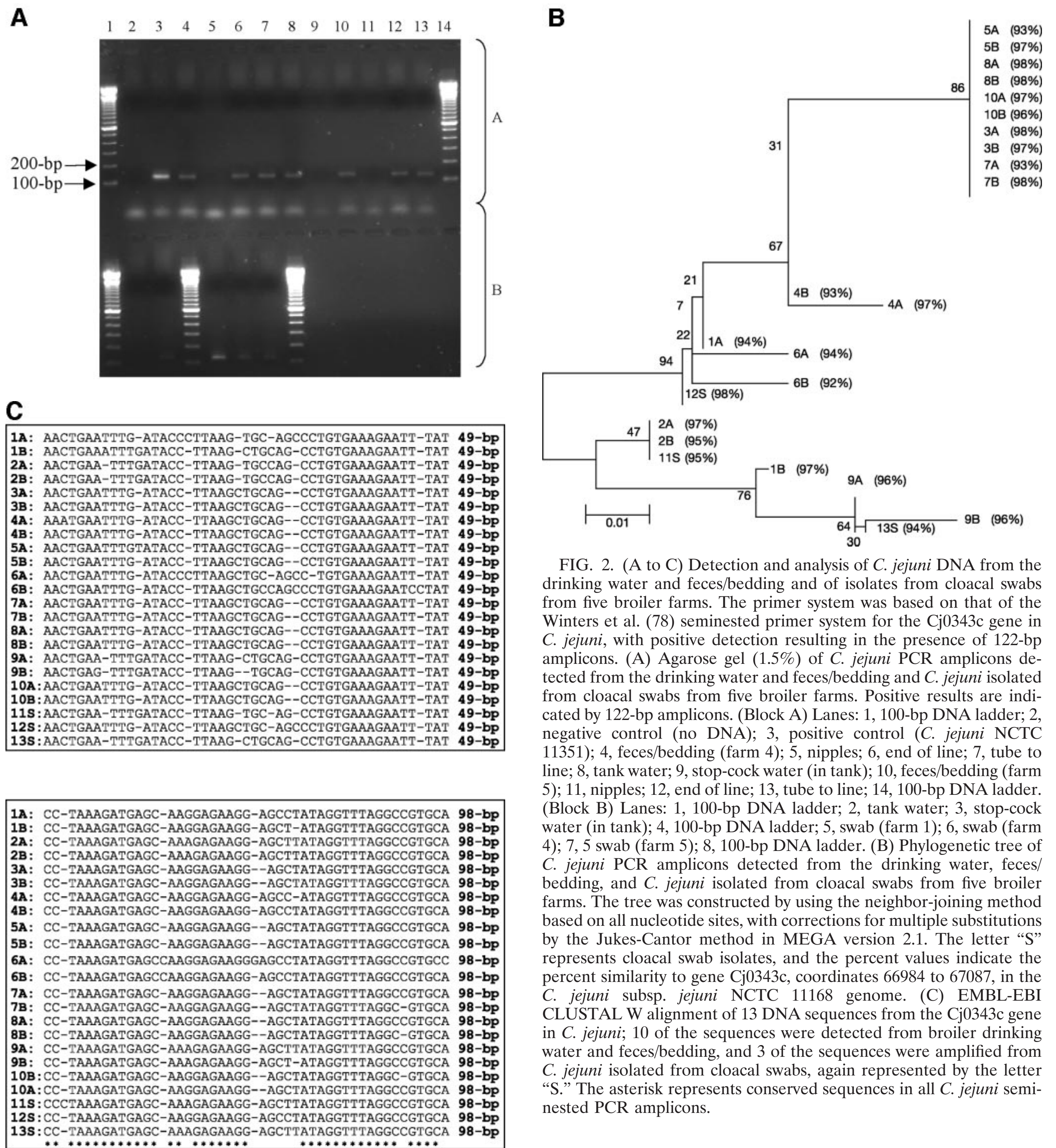

FIG. 2. (A to C) Detection and analysis of *C. jejuni* DNA from the drinking water and feces/bedding and of isolates from cloacal swabs from five broiler farms. The primer system was based on that of the Winters et al. (78) seminested primer system for the Cj0343c gene in *C. jejuni*, with positive detection resulting in the presence of 122-bp amplicons. (A) Agarose gel (1.5%) of *C. jejuni* PCR amplicons detected from the drinking water and feces/bedding and *C. jejuni* isolated from cloacal swabs from five broiler farms. Positive results are indicated by 122-bp amplicons. (Block A) Lanes: 1, 100-bp DNA ladder; 2, negative control (no DNA); 3, positive control (*C. jejuni* NCTC 11351); 4, feces/bedding (farm 4); 5, nipples; 6, end of line; 7, tube to line; 8, tank water; 9, stop-cock water (in tank); 10, feces/bedding (farm 5); 11, nipples; 12, end of line; 13, tube to line; 14, 100-bp DNA ladder. (Block B) Lanes: 1, 100-bp DNA ladder; 2, tank water; 3, stop-cock water (in tank); 4, 100-bp DNA ladder; 5, swab (farm 1); 6, swab (farm 4); 7, 5 swab (farm 5); 8, 100-bp DNA ladder. (B) Phylogenetic tree of *C. jejuni* PCR amplicons detected from the drinking water, feces/bedding, and *C. jejuni* isolated from cloacal swabs from five broiler farms. The tree was constructed by using the neighbor-joining method based on all nucleotide sites, with corrections for multiple substitutions by the Jukes-Cantor method in MEGA version 2.1. The letter "S" represents cloacal swab isolates, and the percent values indicate the percent similarity to gene Cj0343c, coordinates 66984 to 67087, in the *C. jejuni* subsp. *jejuni* NCTC 11168 genome. (C) EMBL-EBI CLUSTAL W alignment of 13 DNA sequences from the Cj0343c gene in *C. jejuni*; 10 of the sequences were detected from broiler drinking water and feces/bedding, and 3 of the sequences were amplified from *C. jejuni* isolated from cloacal swabs, again represented by the letter "S." The asterisk represents conserved sequences in all *C. jejuni* seminested PCR amplicons.

Cj0343c in *C. jejuni* subsp. *jejuni* NCTC 11168 (Fig. 2B). At farm 4 the same strain of *C. jejuni* detected in tank water (6A and 6B) may have colonized broilers (12S; Fig. 2B), and in farm 5 the same strain of *C. jejuni* that was detected in the tube to the line (9A and 9B) may have also colonized broilers (13S; Fig. 2B). Collectively, this genome-localized evidence suggests that the *C. jejuni* isolated from cloacal swabs taken from farms 1, 4, and 5 were the same as those isolated from water systems of these farms.

Detection of protozoa in all broiler drinking water systems. The same water samples that were screened for the presence of *Campylobacter* were also analyzed for eukaryotic microbes. After collection of 510-ml samples, each sample was subjected to brief screening by bright-field light microscopy using simple wet mounts. The presence of flagellate and ciliate protozoa was confirmed, although further microscopic identification was

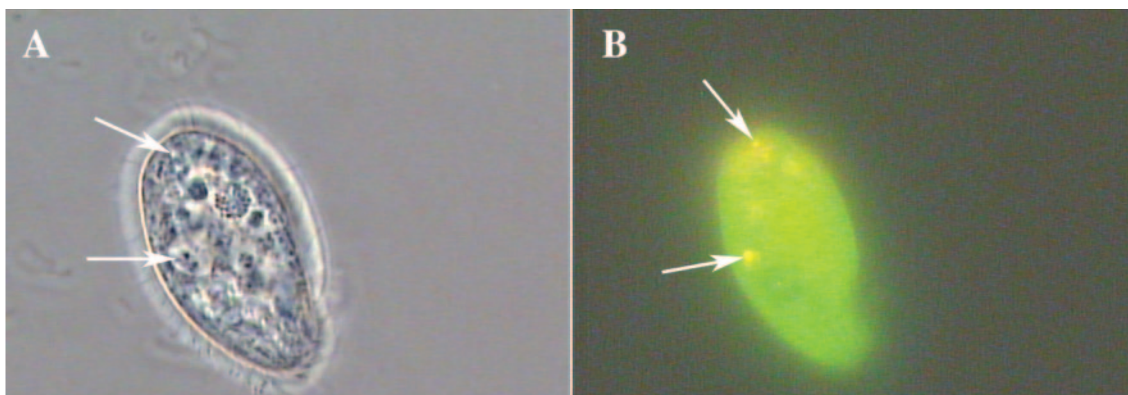

FIG. 3. (A and B) Microscopy of *T. pyriformis* (CCAP 1630/14A) after 24 h of coculture with *C. jejuni* NCTC 11351 in PAS at 25°C. *C. jejuni* was stained with Baclight viability dye before coculture (1:1) with *T. pyriformis*. Magnification, ×100. The arrows indicate *T. pyriformis* vacuoles containing viable and intact *C. jejuni*. (A) Bright-field image of an intact *T. pyriformis* cell cocultured with *C. jejuni*. (B) Fluorescent image of viable (green) *C. jejuni* inside *T. pyriformis*.

not performed. Instead of using the highly skilled and potentially subjective process of protozoa microscopy identification (53, 54, 55), eukaryotic identification was based on the most powerful approach to explore microbial diversity, the analysis of cloned rRNA ribosomal gene sequences using the primer system of Einsele et al. (18, 47). Eukaryotic microorganisms were detected in all water and fecal/bedding samples from all farms, based upon the presence of a 500-bp band in all PCR samples (data not shown). All of these PCR products were cloned and sequenced (Table 3). A wide diversity of eukaryotic microbes were detected in broiler drinking water, i.e., protozoa, uncultured eukaryotic clones, and yeasts and molds (Table 3). Species of protozoa which were detected were the chrysophyte (two flagella) flagellates *Paraphysomonas butcheri* (5- to 20-μm body length; farm 2) and *Spumella oblique* (5- to 20-μm body length; farm 3), and the ciliates *Paraurostyla weissei* (farm 1) and *Aspidisca steini* (20- to 100-μm body length; farm 5), which are all generally reported to be widely distributed in freshwater (53, 54, 55). *Aspidisca* is commonly isolated from freshwater and are small, flattened cells, moving over immersed surfaces with their ventral mouths, usually consuming individually attached particles, e.g., bacteria (54, 55). The majority (73.5%) of clones were yeasts and molds, e.g., *Trichosporon montevideense*, which are generally distributed widely in nature, especially in soils (5, 17, 33, 34, 38, 40–42, 47, 59, 61, 69). The uncultured alveolate clone LEMD251 was detected from farms 1, 3, and 5, while an unidentified eukaryotic 18S rRNA clone LKM67 was detected in the tank of farm 4. Alveolates are a vast group of protozoa comprising of ciliates, dinoflagellates, and apicomplexa characterized by the possession of a system of abutting sacs (alveoli) underlying the cell surface (13, 46, 51). The most divergent of known eukaryotic lineages in phylogenetic trees is represented by anaerobic or aerotolerant organisms, the inhabitants of anoxic (low-oxygen) environments (7, 13). Broiler drinking water systems provide low-oxygen or anoxic habitats for microbes, explaining the detection of anoxic unculturable alveolate clones. Although reports of intracellular prokaryotes in rumen ciliate protozoa (anoxic environment) exist, the potential of anoxic uncultured alveolate clones to act as *Campylobacter* hosts is unknown but nevertheless may exist (9, 43, 44). Protozoa were detected in the water systems of all farms, thus indicating the potential for *Campylobacter* and protozoa to interact.

Internalization of *Campylobacter* in protozoa. *Campylobacter* and protozoa were shown to coexist in poultry water systems. The potential interactions between the two were investigated

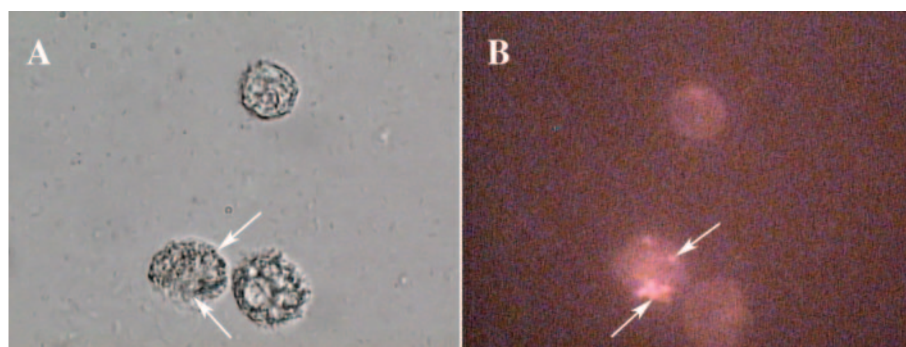

FIG. 4. (A and B) Microscopy of *A. castellanii* (CCAP 1501/10) after 3 days of coculture with *C. jejuni* NCTC 11351 in PAS at 25°C. *C. jejuni* was stained with Baclight viability dye before coculture (1:1) with *A. castellanii*. magnification, ×40. The arrows indicate *A. castellanii* vacuoles containing dead *C. jejuni*. (A) Bright-field image of an intact *A. castellanii* cell cocultured with *C. jejuni*. (B) Fluorescent image of dead (red) *C. jejuni* inside *A. castellanii*.

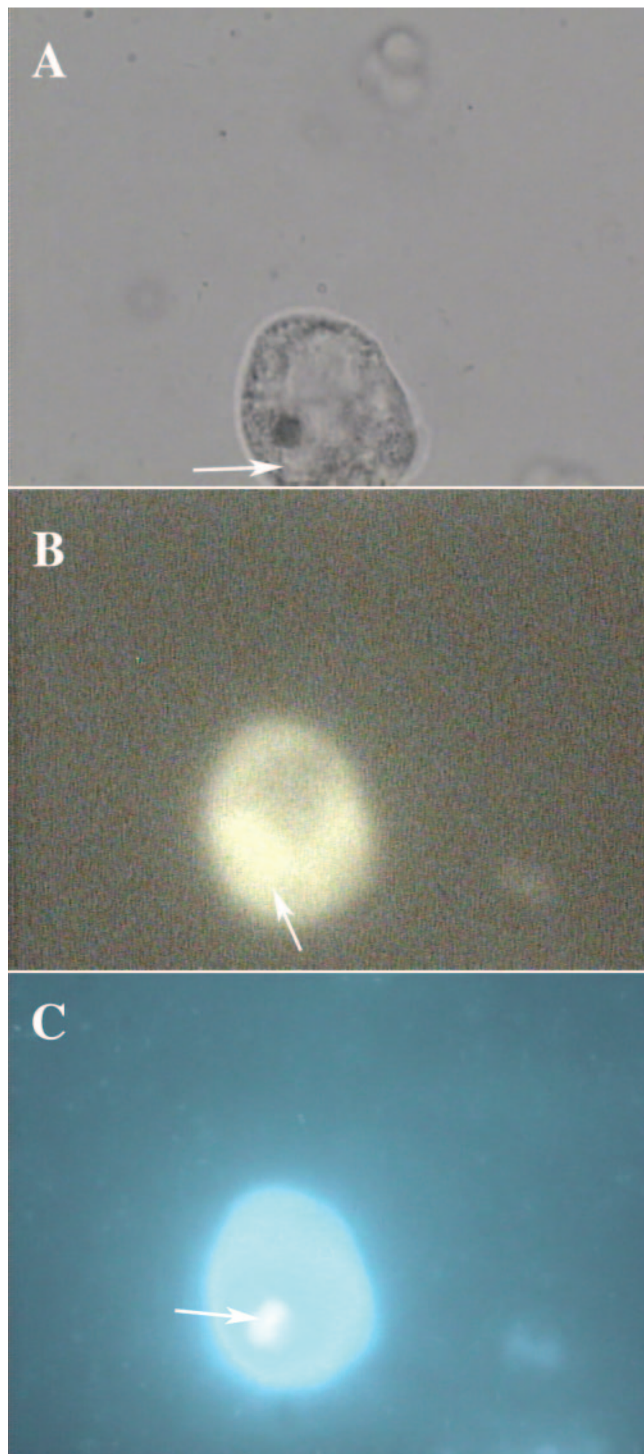

FIG. 5. (A through C) Microscopy of *T. pyriformis* (CCAP 1630/14A) after 3 h of coculture with *C. jejuni* NCTC 11351 at 25°C. *C. jejuni* was stained with FITC-labeled rabbit antibody before coculture (1:1) with *T. pyriformis*. magnification, ×100. (A) Bright-field image of an intact *T. pyriformis* cell cocultured with *C. jejuni*. (B) Fluorescent image of *C. jejuni* inside *T. pyriformis*. The arrow indicates *T. pyriformis* vacuoles containing *C. jejuni*. (C) Intact *T. pyriformis* stained with DAPI after 3 h. DNA (especially in the nucleus) was stained blue with DAPI, and the arrow indicates a *T. pyriformis* nucleus. *C. jejuni*, also stained blue, can be seen outside around the intact *T. pyriformis* cell.

in a series of in vitro experiments by using established model systems. *T. pyriformis* and *A. castellanii* are two types of bacterivorous protozoa commonly observed in surface water that can be grown axenically; studies have shown these bacteria to be subject to infection by bacteria, and both protozoa therefore provide good in vitro models (37, 62). In fluid media, axenically grown *T. pyriformis* and *A. castellanii* ingest nutrients through food vacuole formation (49, 74). In the presence of bacteria, *T. pyriformis* and *A. castellanii* contain digestive food vacuoles containing live bacteria, which for a period of time stay undamaged and can be observed microscopically (64). The use of light microscopy to study protozoa ingesting bacteria has the inherent difficulty of discriminating between bacteria that are bound to the external surface of cells and those that are internalized by them (15). An uncomplicated and inexpensive method for studying phagocytosis uses FITC-labeled bacteria and DAPI as a quenching agent, allowing the simultaneous viewing of intracellular and extracellular bacteria and providing the ability to discriminate between them (31, 60, 75). The bacterial viability assay clearly showed viable (green) and after longer incubation times (3 days) dead (red) *Campylobacter* inside food vacuoles of *A. castellanii* and *T. pyriformis* (Fig. 3 and 4, respectively). The final proof that *Campylobacter* were inside protozoa was provided by the DAPI/FITC method, wherein *C. jejuni* were clearly visible inside *T. pyriformis* (Fig. 5A to C). Internal *Campylobacter* were stained an extremely bright green color (FITC) (Fig. 5B), while external *Campylobacter* and protozoan DNA (especially in the nucleus) were stained blue with DAPI (Fig. 5C).

Delayed decline of *Campylobacter* viability in cocultures. The effect of *A. castellanii* and *T. pyriformis* on the *Campylobacter* viability at 25°C in PAS was examined. When all strains of *Campylobacter* were incubated with protozoa, the number of recovered viable *Campylobacter* decreased (Fig. 6A to C). No significant ($P > 0.05$) differences were noted between *Campylobacter* strain viability during the first 24 h (Fig. 6A). During the first 3 h slightly more viable *Campylobacter* were recovered from planktonic suspensions than from suspensions of *Campylobacter* and protozoa (Fig. 6A). This may have been because the protozoa were feeding at a high rate after being starved. Initially fewer bacteria were recovered from cocultures containing *T. pyriformis* than *A. castellanii*, probably because *T. pyriformis* moves at a much higher rate and would be expected to be a more active bacterial predator (9, 37, 43, 44). After 5 days more notable differences existed between the *Campylobacter* viable counts (Fig. 6B and C). For example, after 5 days the number of viable *C. jejuni* organisms (poultry isolate) recovered from coculture with *A. castellanii* was significantly ($P < 0.05$) higher than the numbers of recovered planktonic *C. jejuni* NCTC 11351, *C. jejuni* (poultry isolate), and *C. coli* NCTC 11366 (Fig. 6C). After 5 days, significantly less ($P < 0.05$) planktonic *C. coli* NCTC 11366 and *C. jejuni* NCTC 11351 were recovered compared to the *C. jejuni* (poultry isolate) in coculture with *T. pyriformis* (Fig. 6B).

The most important overall viability finding was that the *C. jejuni* strains both remained viable for significantly ($P < 0.05$) longer (an extra 36 h, i.e., from 7.75 to 9.25 days) when they were incubated with both strains of protozoa (Fig. 6B and C). The presence of *T. pyriformis* did not significantly increase ($P > 0.05$) the time required for a complete decline in *C. coli*

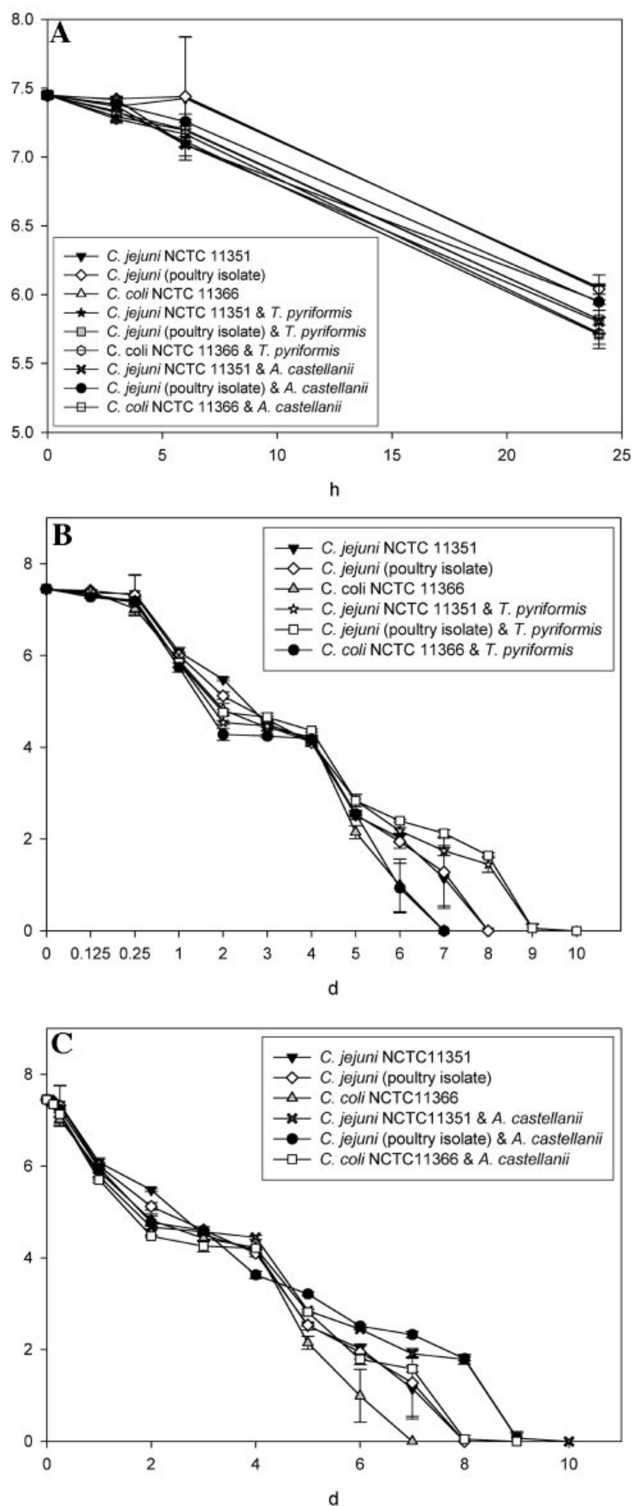

FIG. 6. (A through C) Survival of *Campylobacter jejuni* NCTC 11351, *C. coli* NCTC 11366, and *C. jejuni* subsp. *jejuni* (poultry isolate) when cocultured with *A. castellanii* (CCAP 1501/10) and *T. pyriformis* (CCAP 1630/14A). Cocultures (1:1 ratio of *Campylobacter* to protozoa) and planktonic strains of *Campylobacter* were prepared in PAS solution and incubated at 25°C for up to 10 days, when viable *Campylobacter* were no longer obtained. The results are presented as average (performed in quadruplicate) viable *Campylobacter* recovered per ml from PAS, and error bars indicate the standard deviations. (A) Viable counts of recovered *Campylobacter* during the first 24 h of incubation from cocultures with *A. castellanii* and *T. pyriformis*. (B) Viable counts of recovered *Campylobacter* during 10 days of incubation from cocultures containing *T. pyriformis*. (C) Viable counts of recovered *Campylobacter* during 10 days of incubation from cocultures containing *A. castellanii*.

viability. However, the decline of *C. coli* viability was significantly ($P < 0.05$) delayed during coculture with *A. castellanii*, i.e., 8 days (Fig. 6C). This suggests that the relationship between *Campylobacter* and protozoa is strain specific. Tezcan-Merdol et al. (72) found that the uptake and replication of different serovars of *Salmonella enterica* varied greatly within *Acanthamoeba* spp., suggesting that *Acanthamoeba* spp. can differentiate between serovars of salmonellae.

Increased *Campylobacter* disinfection resistance in cocultures. When protozoa were 3 and 6 days old before coculturing, *Campylobacter* internalized by protozoa were significantly more resistant to disinfection ($P < 0.05$) than purely planktonic *Campylobacter*, which were all killed by the Virudine (Table 4 and Fig. 7). Significantly more ($P < 0.05$) internalized campylobacters survived when *T. pyriformis* and *A. castellanii* used in coculture were grown for 3 and 6 days than for 9 days, with maximal *Campylobacter* survival when protozoa were 3 days old (Table 4). After coculture with both *T. pyriformis* and *A. castellanii*, slightly more internalized *C. jejuni* subsp. *jejuni* (poultry isolate) survived disinfection than *C. jejuni* NCTC 11351 and *C. coli* NCTC 11366; however, these differences were not significant ($P > 0.05$) (Table 4).

After 3 h of coculture time, significantly less ($P < 0.05$) internalized *C. jejuni* NCTC 11351 survived disinfection within *A. castellanii* than *T. pyriformis* (Fig. 7). However, after 6 h of coculture time, the amount of viable *C. jejuni* was not significantly different between both kinds of protozoa ($P > 0.05$) (Fig. 7). Maximum survival of internalized *C. jejuni* NCTC 11351 occurred after 12 h of coculture time with both protozoa, with significantly more ($P < 0.05$) *C. jejuni* surviving within *A. castellanii*. After 24 h of coculture time the amount of recovered viable *C. jejuni* NCTC 11351 had significantly dropped ($P < 0.05$), and the disinfection survival of internalized *C. jejuni* was no longer significantly different ($P > 0.05$) between *T. pyriformis* and *A. castellanii*. Collectively, these data suggest that the age or growth phase of protozoa and the interaction time (coculture time) greatly affects internalized *Campylobacter* disinfection resistance.

DISCUSSION

Protozoa detected in broiler drinking water systems. Despite the presence of disinfectant, low temperatures, and flow regimes in drinking water distribution systems, the growth and persistence of bacteria in is well documented, and the diversity of protozoa and number of organisms are usually a function of the amount of available organic matter, including bacterial load (11, 53). Poultry drinking water systems allow sediment accumulation on pipe walls and tanks (Fig. 1), partly explaining the wide diversity of eukaryotes that was detected in the water systems (11, 53). The central role of protozoa in aquatic food webs as major grazers of phytoplankton and bacteria is firmly

TABLE 4. Effect of age of protozoa during coculture on *Campylobacter* disinfection resistance[a]

Protozoan age (days)	Location	Avg *Campylobacter* count (CFU/ml) ± SD		
		C. coli NCTC 11366	*C. jejuni* NCTC 11351	*C. jejuni* (poultry isolate)
T. pyriformis				
3	Internal	$(3.52 \times 10^3) \pm 285$	$(4.28 \times 10^3) \pm 730$	$(5.77 \times 10^3) \pm 999$
3	External	0 ± 0	132 ± 18	313 ± 22
6	Internal	$(11.21 \times 10^2) \pm 134$	$(3.25 \times 10^3) \pm 164$	$(4.56 \times 10^3) \pm 644$
6	External	5 ± 3	16 ± 10	33 ± 10
9	Internal	0 ± 0	0 ± 0	0 ± 0
9	External	0 ± 0	0 ± 0	0 ± 0
A. castellanii				
3	Internal	269 ± 33	326 ± 32	375 ± 16
3	External	1 ± 1	3 ± 1	5 ± 1
6	Internal	113 ± 12	154 ± 24	172 ± 20
6	External	0 ± 0	0 ± 0	0 ± 0
9	Internal	0 ± 0	0 ± 0	0 ± 0
9	External	0 ± 0	0 ± 0	0 ± 0

[a] *T. pyriformis* (CCAP 1630/14A) and *A. castellanii* (CCAP 1501/10) were each grown for 3, 6, and 9 days before being cocultured for 3 h at 25°C (1:1 ratio) with *Campylobacter* spp. in PAS solution. A 1:1,000 dilution of Virudine (1-min contact time) was then used to kill planktonic *C. jejuni*, followed by neutralization using STS, gravity filtration (0.8-μm pore size), rinsing and resuspension in PAS, sonication (10 s at 40 W), and counts of viable *Campylobacter*. Results are presented as the average (performed in quadruplicate) numbers of viable *Campylobacter* recovered ml of PAS.

established, and species of protozoa detected in water systems were the flagellates *Paraphysomonas butcheri* and *Spumella oblique* (ingest bacteria) and the ciliate *Aspidisca steini*, which are frequently isolated from freshwater sources (23, 54, 55, 71, 73). Broiler drinking water contained a variety of protozoa; thus, it is concluded that poultry drinking water systems have strong potential to support and facilitate biological interactions between bacteria and protozoa.

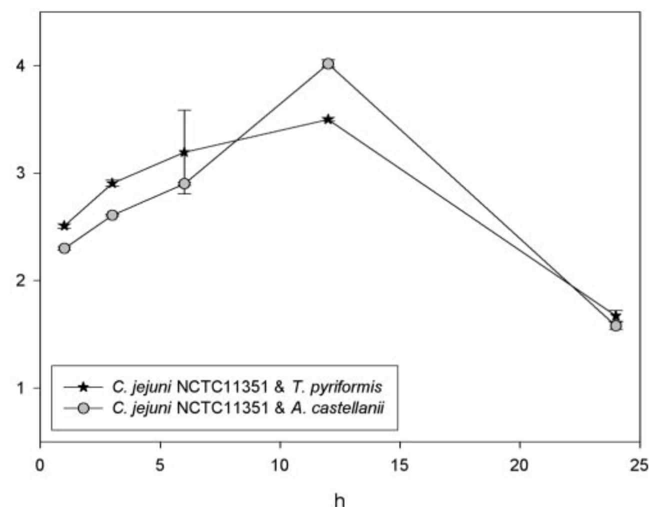

FIG. 7. Effect of coculture time on the survival of *C. jejuni* NCTC 11351 during disinfection. Cocultures with *C. jejuni* and *A. castellanii* (CCAP 1501/10) and *T. pyriformis* (CCAP 1630/14A) (1:1 ratio of *Campylobacter* to protozoa) were prepared in PAS solution and incubated for up to 24 h at 25°C. After 3, 6, 9, 12, and 24 h, a 1:1,000 dilution of Virudine (1-min contact time) was used to kill planktonic *C. jejuni*, followed by neutralization with STS, gravity filtration (0.8 μm), rinsing and resuspension in PAS, sonication (10 s at 40 W), and viable *Campylobacter* counts. The results are presented as average (performed in quadruplicate) viable *Campylobacter* recovered per ml of PAS, and error bars indicate the standard deviations.

***Campylobacter* and protozoan epidemiology.** The strains of *C. jejuni* present in water systems also appeared to colonize broilers as on farms where *C. jejuni* was detected in water; the organism was also found in broilers and feces/bedding. Previous farm epidemiology studies looking at a variety of potential reservoirs have shown that common strains can be found across a number of hosts. On et al. (50), using pulsed-field gel electrophoresis, found evidence of identical *C. jejuni* clones infecting humans, poultry, and cattle. Water plays an important role in the ecology of *C. jejuni*, and it can enter drinking water distribution systems through the fecal contamination of untreated ground or surface water, treatment failure, or distribution system failure (4, 25). Providing water is of low turbidity, standard chlorination procedures are normally sufficient to prevent the spread of planktonic campylobacters along water mains (12, 25, 79). Protozoa and *C. jejuni* were both detected in the drinking water systems of intensively reared poultry, highlighting the strong potential of protozoan-*Campylobacter* interactions. The detection of protozoa, including heterotrophic flagellates which are characteristically phagotrophic and are quantitatively the most important consumers of other microbes, means that such protozoa in the water supplies of broiler farms would probably ingest *Campylobacter* (3, 6, 23, 70).

***Campylobacter* internalization and viability decline.** The implications to the poultry industry of our in vitro coculture assays are greatly increased by the detection of ciliates closely related to *T. pyriformis* and by the usage of *A. castellanii*, the most commonly used protozoa in in vitro coculture assays. The studies reported here provide new evidence of the ability of *Campylobacter* to survive in the presence of protozoa. The three microscopic methods used to examine cocultures provided images where *Campylobacter* was clearly observed within

vacuoles of *A. castellanii* and *T. pyriformis*. The viability coculture study, which examined the effects of the presence of protozoa toward *Campylobacter* viability, revealed new data with important implications for the broiler industry. In in vitro conditions, the presence of protozoa can significantly ($P < 0.05$) delay the decline in *C. jejuni* viability for up to 36 h at temperatures at which broilers are reared (25°C), thus potentially increasing the risk of *Campylobacter* colonization of broilers. This could be because *Campylobacter* released from protozoa undergo phenotypic changes, becoming more resistant to low-nutrient conditions and temperatures at which their decrease in viability is more rapid than at lower temperatures, e.g., 4°C (10, 15). When the broilers from the farms were 17 days old they were given a vaccine for infectious bursal disease in their drinking water. For this vaccine to be effective, the chlorine must be removed from the water. This method of administration of the infectious bursal disease vaccine could actually infect broilers with *C. jejuni*. *Campylobacter* would be expected to be periodically released from the protective environment of protozoa. However, during this vaccination process, the now planktonic *Campylobacter* would be in unchlorinated drinking water; thus, there would be a higher potential for infecting broilers. The timing with which the vaccine is administrated may be crucial because broilers are 17 days old and soon after this age, i.e., 3 weeks, they start to be infected with *Campylobacter*.

In broiler water supplies many other factors would affect *Campylobacter* viability. For this research, 1:1 ratios of protozoa to *Campylobacter* in cocultures were used. In water systems the concentrations of protozoa and *Campylobacter* would vary between farms and also within the different sections of water drinking systems in the same farm. In addition to nonliving organic matter, many varieties of eukaryotic and prokaryotic microbes also exist in a planktonic state and/or within biofilms. There would also be much greater variation in the physiological status, e.g., age and nutrient availability, of microbes in broiler drinking water. Further studies examining the effects of coculturing different *Campylobacter* strains (and other bacteria) with *T. pyriformis*, *A. castellanii*, and flagellates in cocultures, with variations in the ages of the bacteria and protozoa, would prove interesting.

***Campylobacter* disinfection resistance.** To our knowledge this is the first report of *Campylobacter* within protozoa demonstrating resistance to a disinfectant widely used in the poultry industry. Other novel areas of this research included examinations of the effects of the growth phase of the protozoa used in coculture and of the coculture time on *Campylobacter* disinfection resistance. *Campylobacter* in the presence of *T. pyriformis* and *A. castellanii* was significantly more ($P < 0.05$) resistant to disinfection when the protozoa used for coculture were grown for 3 and 6 days. The presence of cellulose in the cyst walls of *Acanthamoeba* spp. is a unique factor that may contribute to their disinfectant resistance, providing a physical barrier protecting them from extremes in pH and temperature, desiccation, anoxia, and antibiotics and disinfectants (76). The LuxS gene of *C. jejuni* 11168 produces the functional signal autoinducer 2 (AI-2), which is responsible for quorum sensing (19). As well as physical protection from external stresses, bacteria are densely packed in biofilms and/or protozoa. When *Campylobacter* were inside protozoa in food vacuoles, signaling molecules, e.g., AI-2, could have been present at higher concentrations than in equal numbers of there planktonic counterparts, potentially resulting in increased stress resistance. The exposure of protozoa to free iodine residuals may disrupt lysosomal hydrolase activity, delaying bacterial digestion (37). Undigested, viable bacterial cells may remain inside *A. castellanii* and *T. pyriformis* due to hydrolase disruption for up to 24 h after chlorine exposure, which killed planktonic bacteria (37).

King et al. (37) performed the only other major study examining the survival of bacterial pathogens within *T. pyriformis* and *A. castellanii* during disinfection. Similarities in protocol procedure include the use of 24-h-old bacterial strains, 1:1 amounts of protozoa for coculture (both at 10^4 ml^{-1}), and neutralization of a halogen disinfectant with STS (1, 0.5, 0.25, and 0.125 mg of chlorine) after a 1-min contact period. However, King et al. did not differentiate between surviving external and internal bacteria, and the site of bacterial carriage was not unequivocally proven. Other established protozoan-bacterial relationships include *Helicobacter pylori*, one of the world's leading pathogens, colonizing ca. 60% of the global population (52, 77). The major mode of transmission of *H. pylori* remains unknown, and the finding of bacterial DNA in water samples, together with the high infection rate in developing countries suggests that environmental factors, e.g., interaction with amebas, could be involved in its transmission (77). The cocultivation of *H. pylori* with *A. castellanii* circumvented the bacterial requirements for precise microaerophilic conditions and a large supply of nutrients in order to grow, with a 100-fold increase of bacterial counts after 7 days (77). The putative dependence of *H. pylori* on free-living amebas in nature could be important with respect to transmission and prevalence, as has already been shown for *Legionella pneumophila* (1, 36, 37, 64, 76, 77). The potential for waterborne protozoa to act as vehicles for the *Campylobacter* infection of broilers is greatly increased by reports of *H. pylori* growing within *Acanthamoeba*, which was originally assigned taxonomically into the genus *Campylobacter*.

In conclusion, *C. jejuni* and a variety of protozoa were detected in broiler houses. In vitro, the presence of *T. pyriformis* and *A. castellanii* can significantly delay the decline of *Campylobacter* viability and significantly increase *Campylobacter* resistance to industrial disinfection. Collectively, these findings strongly suggest that the presence of protozoa and their interaction with *Campylobacter* in the water supplies of intensively reared poultry greatly increases the potential of broilers being colonized with *Campylobacter*. Viable pathogenic bacteria residing in protozoa present a new challenge in terms of disease control and sanitation of contaminated water sources since disinfectant efficiency is based upon planktonic tests.

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