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Research Article

Persistence of Two *Campylobacter jejuni* Strains in Soil and on Spinach Plants

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There are indications that the more frequent use of untreated organic residues for fertilization results in increased risk of contamination with human pathogens. Here, we evaluate the ability of two different strains of *Campylobacter jejuni* to persist in manure and soil as well as spread to spinach plants. It was revealed that different strategies for inoculation of *C. jejuni* contribute to the persistence of the bacterium in soil, roots, and shoots. Upon inoculation of the bacteria into manure prior to soil application, the amount of *C. jejuni* subsequently recovered in soil was higher than that from treatments involving the addition of *C. jejuni* cells to the soil after plant emergence. Irrespective of the bacterial inoculation dose and strategy employed, the *C. jejuni* content in soil remained relatively constant, whereas the majority of *C. jejuni* cells applied to spinach leaves could be recovered during the whole evaluation period of 21 days.

1. Introduction

In contrast to infections by *Salmonella* spp. and pathogenic *Escherichia coli* that cause massive foodborne outbreaks, campylobacteriosis is mainly presented as sporadic illness [1]. In view of its sporadic nature combined with an unusual microaerophilic and thermophilic lifestyle, recovery of *Campylobacter* spp. outside its host is a major challenge, predominantly resulting in unidentified point sources of *Campylobacter* contamination [2]. One possible means of *Campylobacter* entry into the human food chain is through application of untreated animal manures and/or biosolids to agricultural crop land. Organic manure is an important source of plant nutrients and organic matter, particularly within organic farming where no mineral fertilizers are allowed. The risk for *Campylobacter* contamination of crops is highest in cases where the produce is likely to be eaten raw, including crops such as salads, spinach, fruit, and various vegetables. Consistently, *Campylobacter* spp. has been detected on produce sampled at the marketplace, such as spinach, lettuce, radish, green onion, potatoes, and parsley [3], carrots and cabbage [4], mixed salad vegetables [5], mushrooms [6], and spinach and fenugreek [7]. The extent of *Campylobacter* spp. survival in manure is affected by the type of animal, their diet stress, and age [8, 9], respectively, as well as manure management and method of application [10, 11]. The majority of studies performed to date indicate that *Campylobacter* species are not able to persist effectively in solid manure once excreted [12–17]. However, contrasting results have been obtained in the most recent investigation, which shows that *Campylobacter* cells survive in excreted cattle feces for long periods in compost [18]. Regardless of the rate of decline of *Campylobacter* species in manure, the bacteria might potentially be able to survive and proliferate in the rhizosphere after application of manure to soil, since this site is considered a reservoir of human pathogens [19]. Moreover, root colonization may lead to endophytic spread from roots to shoots of some pathogens [20], thus representing an even greater source of infection.

To determine the risks connected to the persistence of *Campylobacter* in crop produce in the presence of the indigenous microflora, the ability of these bacteria to colonize manure, soil, and plant parts required thorough evaluation. However, despite the difficulties in isolating and

quantifying *Campylobacter* spp. in substrates containing complex microbial communities, limited molecular detection and/or quantification techniques have been applied for exploring their persistence in manure, soil, and on or within plant products. The overall objective of the present study was to compare the abilities of two *C. jejuni* strains inoculated at different concentrations in manure and soil to spread further through the rhizosphere to spinach root and shoot tissue. Moreover, quantitative differences in *C. jejuni* among the sites investigated (soil, roots and shoots) were evaluated by means of molecular targeting.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. We employed the completely genome sequenced [21, 22] bacterial strains *C. jejuni subsp. jejuni* CCUG 6824 (NCTC 11168) and *C. jejuni subsp. jejuni* 81116 (NCTC 11828) in this study. The strains were grown and maintained on mCCDA-Preston agar plates (Oxoid LTD., Basingstoke, Hampshire, England) supplemented with CCDA selective supplement (Oxoid LTD., Basingstoke, Hampshire, England) for 48 h, followed by transfer to Bolton-selective enrichment broth (Merck KgaA, Darmstadt, Germany). Plates inoculated with *Campylobacter* were incubated at 42°C under microaerophilic conditions generated by an activated BBL CampyPak plus gas generator envelope placed in a BBL GasPak Jar system (BBL Microbiology Systems, Cockeysville, USA). Liquid cultures were incubated at 37°C under microaerophilic conditions (GasPak EZ Campy Container System, Becton Dickinson, Sparks, USA) for 48 h. Subsequently, 10 mL of culture was transferred to fresh Bolton broth and reincubated at 37°C under similar conditions for 24 h until OD_{600} measured approximately 0.15, corresponding to the early exponential phase. For inoculation of manure, *Campylobacter* bacteria were harvested, washed 3 times with 0.9% NaCl, and resuspended in 0.9% NaCl.

2.2. Manure and Soil. Cattle slurry (dry substance: 10.9%, pH: 7.0, Tot-N: 4.3 kg ton^{-1}, NH_4-N: 2.2 kg ton^{-1}, ash content: 2.3%, C/N ratio: 20, P: 0.59 kg ton^{-1}, K: 4.2 kg ton^{-1}, Mg: 0.79 kg ton^{-1}) was collected in a deep pit at an organically managed farm in Sandviken, Sweden, and stored at 4°C until use. Clay loam soil (clay content: 36%, sand content: 19%, humus content: 4.2%, pH: 6.6, dry substance: 88.9%, P: 1.6 mg 100 g^{-1} air-dried soil, K/Mg ratio: 0.3, Ca: 250 mg 100 g^{-1} air-dried soil, N-tot: 33 kg ha^{-1}, NH_4-N: 0.159 mg 100 g^{-1} dry substance, NO_3-N: 1.161 mg 100 g^{-1} dry substance) was collected at a biodynamic farm in Järna, Sweden, and stored at 4°C until use. Soil was collected from a 1 × 1 m square at a depth of approximately 20 cm, sieved (2 mm) and mixed prior to use. Chemical analyses were performed by Eurofins Laboratories (Kristianstad, Sweden).

2.3. Bacterial Inoculation of Manure and Soil. Two separate experiments were performed. Experiment A examined the effect of inoculum size on the colonization ability of *C. jejuni* 6824 inoculated into manure before soil application. In experiment B, we investigated the potential differences in survival in soil and plant material between *C. jejuni* 6824 and *C. jejuni* 81116. Organically produced spinach seeds (variety Gamma) were used in both experiments and grown in plastic pots (6.5 × 6.5 × 5 cm). Each treatment was replicated 5 times and sampled at 4 specific dates, leading to a total of 20 pots per treatment.

In experiment A, three inoculation doses of *C. jejuni* 6824 were used, specifically, 10^5, 10^6, and 10^7 CFU g^{-1} slurry (corresponding to 10^4, 10^5, and 10^6 CFU g^{-1} soil), along with a control containing only 0.9% NaCl buffer. In general, 220 mL slurry was inoculated with 22 mL bacterial suspension or 0.9% NaCl buffer and mixed with 3 kg of soil. Individual pots received 130 g of this mixture, and 6 spinach seeds were sown in each pot at a depth of approximately 2 cm.

Pots in experiment B contained 130 g of the soil-manure mixture described above, but no *Campylobacter* bacteria. When plants were 14 days old, 10 mL bacterial suspensions (each containing one of the *Campylobacter* strains at a concentration of ~ 1 × 10^7 CFU mL^{-1}) were carefully added with a pipette to the soil and the lowest 2 cm of the shoots in each pot.

2.4. Cultivation and Sampling of Spinach. Pots containing the spinach plants were placed in a phytotron at SLU, Uppsala. Conditions were set to match those in Swedish fields in June and July, specifically, a light/dark cycle of 18 h/6 h, temperatures of 20°C/12°C, relative humidity of 70%, and light intensity of 400 μmol·m^{-2}·s^{-1}. In experiment A, pots were sampled at 7, 14, 21, and 28 days following inoculation of *Campylobacter*, whereas those in experiment B were sampled at 1, 7, 14, and 21 days post inoculation.

2.5. DNA Extraction. The plants in experiments A and B were removed and the soil in each pot was mixed before an aliquot (10 g) was removed and stored at −20°C, prior to grinding with a mortar and DNA extraction. From each sample, 500 mg soil was used for extraction with the FAST DNA soil kit (MP Biomedicals). Plant roots and shoots were separated, and the roots were thoroughly washed in sterile water to remove soil particles and bacterial cells not firmly attached to the roots. For the root and shoot samples, various amounts (between 100 and 400 mg) were used for DNA extraction. These differences were considered when analyzing the data.

2.6. Quantification of Campylobacter mapA Genes Using Real-Time PCR. Real-time PCR was employed to estimate the quantitative differences between *C. jejuni* present within soil and on plant roots and shoots, respectively, using the taxa-specific primers QCjmapANF (5′-GGTTTTGAAGCAAAG-ATTAAAGG- 3′) and QCjmapANR (5′-AAGCAATAC-CAGTGTCTAAAGTGC- 3′) targeting the *mapA* gene [23]. Gene abundance was determined in three independent DNA extracts from individual samples, and three nontemplate controls were included in each PCR assay. Real-time PCR reactions were performed in 20 μL mixtures containing 1x

Flash SYBR Green q-PCR master mix (Finnzymes, Finland), 1x Rox reference dye (Finnzymes), 0.5 μM each primer and 20 ng genomic DNA from the soil/roots as template. The following thermal cycling conditions were employed for amplification: 95°C for 15 min, 40 cycles of 94°C for 15 s, followed by 58°C for 30 s and at 72°C for 30 s. The melting curve data was collected using a span between 55 and 95°C with 0.5°C increments and 10 s dwell time. Standard curves were obtained using serial dilutions of genomic DNA from the strain CCUG 6824. DNA concentrations were determined using spectrophotometry (Nanovue, GE Healthcare). The standards contained between 3×10 and 3×10^5 *Campylobacter mapA* gene copies per μL of sample calculated directly from the measured DNA concentration and genome size of the sequenced *C. jejuni* 6824 strain. Since *Campylobacter* spp. typically consists of a single *mapA* operon [23], the *mapA* copy number should be equivalent to bacterial cell number. To confirm the absence of potential PCR inhibitors, genomic DNA, in combination with extracted soil/root DNA, was quantified and compared with the resulting gene copy numbers of genomic DNA alone. Moreover, soil DNA was diluted, and the different concentrations quantified and analyzed.

2.7. Detection Limits. Soil, root, and shoot materials were inoculated with different dilutions of bacterial suspension containing *C. jejuni* 6824 corresponding to concentrations of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 g^{-1} plant or soil material. For these analyses, DNA was extracted from 500 mg of soil, 200 mg of shoot material, and 100 mg of root samples, as described above. DNA extracts were evaluated with the real-time PCR assay under the above conditions.

2.8. Statistical Analyses. Differences in *mapA* gene copy numbers between treatments and environments were tested for significance using one-way ANOVA and unpaired *t*-test (GraphPad Prism v. 5, GraphPad Software, San Diego, CA, USA). For all analyses, $P < .05$ was considered the level of significance.

3. Results

3.1. Real-Time PCR. Real-time PCR was performed to estimate the quantitative differences in *C. jejuni* amounts among soil samples and plant parts inoculated at different time points with varying bacterial concentrations (experiment A) and strains (experiment B). Different concentrations of *C. jejuni* were used for soil inoculation to determine the lowest possible dose that resulted in a detectable signal. The primers employed in this study were previously designed to amplify *mapA* sequences specific for *C. jejuni* [23]. Extracts of genomic DNA from strain 6824 were used to generate standard curves that allowed analysis of the correlation between the cycle threshold (C_t) and *mapA* gene copy numbers in samples. The real-time PCR setup was very efficient, presenting a linear response (r^2) of >0.99 for genomic DNA of 3×10^1 and 3×10^5 gene copies per microliter of DNA. Specific PCR products were identified using melting curve analysis with a reproducible T_m of 75.5–76.0°C. Standard curves generated using serial dilutions of genomic DNA in water resulted in the linear equation: $y = -3.651x + 38.95$ with $R^2 = 0.998$, which was nearly identical to the linear regression equations for soil, roots, and shoots. These results correspond to detection limits of 10^4 CFU/g for soil, roots, and shoots. Controls without templates resulted in negligible values. The average efficiency was calculated as 87.4%, and standard curves displayed similar slopes between runs (−3.52 to −3.80).

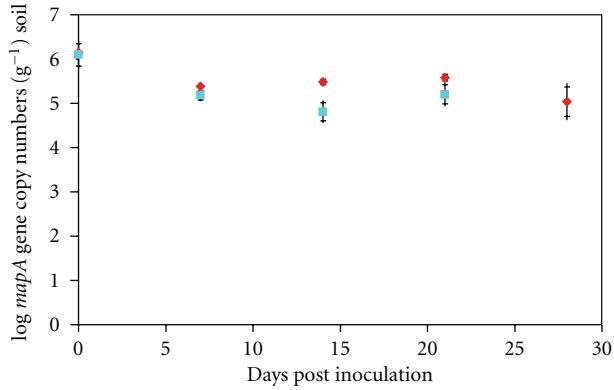

Figure 1: Comparison between the detection of *C. jejuni* strain 6824 *mapA* gene copies in soil following the different inoculation methods in experiments A (red diamonds) and B (turquoise squares). Error bars represent standard deviations from 5 replicate samples and 3 different real-time PCR reactions per replicate.

3.2. Quantification of Campylobacter jejuni in Inoculated Soil. Experiments A and B were performed to (1) compare the spreading patterns of *C. jejuni* 6824 inoculated into manure at different doses prior to application to soil planted with spinach (experiment A) and (2) investigate the potential differences in persistence of two *C. jejuni* strains applied to soil at 14 days after planting of spinach (experiment B). The two distinct stages of bacterial inoculation (i.e., in connection with planting and 14 days after planting) yielded similar initial concentrations of *C. jejuni* cells in soil corresponding to approximately log 6 gene copies g^{-1} soil (Figure 1). On subsequent sampling occasions, slightly higher levels of *mapA* gene copy numbers were detected in soil samples from experiment A, compared to those in experiment B. Inoculation doses of 10^5 and 10^6 *C. jejuni* cells g^{-1} soil led to the detection of *mapA* at all sampling dates in experiment A (Figure 2). Gene levels were 10-fold lower after 4 weeks at the inoculation dose of 10^6 CFU g^{-1} (5.0 log gene copies compared to 6.1 log gene copies g^{-1} soil ($P < .001$)) and 4.7 to 4.3 log gene copies g^{-1} soil ($P < .05$) at the 10^6 cells g^{-1} bacterial inoculation dose. No *mapA* gene copies were detected in the lowest bacterial inculation dose of 10^4 *C. jejuni* cells g^{-1} soil. Consistent with these results, similar patterns were observed in experiment B, with only slightly, reductions in gene levels from 6.1 to 5.2 *mapA* copies g^{-1} soil ($P < .001$) in strain 6824 and 5.9 to 5.4 *mapA* gene copies g^{-1} soil in strain 81116 ($P < .05$) between the first

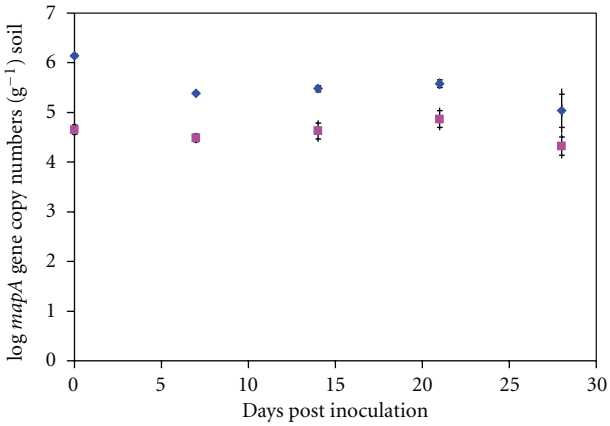

Figure 2: Detection of *C. jejuni mapA* gene copies in soil from experiment A throughout the sampling period of 28 days, with starting inoculation doses of 10^6 and 10^7 *C. jejuni* cells, corresponding to 10^5 (blue diamonds) and 10^6 (pink squares) cells g^{-1} soil. Error bars represent standard deviations from 5 replicate samples and 3 different real-time PCR reactions per replicate.

and last soil sampling dates (Figure 3). In experiment B, we observed a small but significant ($P < .05$) difference in *mapA* gene copy numbers between the two bacterial strains at 7 and 14 days after planting. *C. jejuni* 81116 was detected at levels of 5.6 and 5.1 log gene copy numbers g^{-1} soil, respectively, compared to 5.2 and 4.8 log gene copy numbers g^{-1} soil for strain 6824.

3.3. Detection of Campylobacter jejuni in Spinach Roots. In experiment A, the highest initial bacterial concentration (10^7 cells g^{-1} slurry corresponding to 10^6 cells g^{-1} soil) resulted in detection of *mapA* gene copies in the spinach roots, but not consistently over all 5 replicates. After 7 days, the *mapA* gene was detected in 3 out of 5 replicates with a mean value and standard deviation of 4.6 ± 0.35 log gene copy numbers g^{-1} root material. Upon sampling 14 and 28 days after inoculation, the *mapA* gene was identified in only one replicate with 4.7 log gene copy numbers g^{-1} root material on both occasions. The *mapA* gene was not detected at 21 days after inoculation (data not shown).

Data obtained from experiment B presented another picture whereby *mapA* was detected at all sampling dates in all replicate samples for both *C. jejuni* strains (Figure 4). There were no major differences between the strains, although the gene was slightly more abundant in roots inoculated with strain 81116 at 21 days after inoculation compared to strain 6824, with a *mapA* number of 5.0 in relation to 4.6 log copy numbers g^{-1} root material ($P < .05$).

3.4. Detection of Campylobacter jejuni Associated with Spinach Leaves. In experiment A, no copies of *mapA* were detected in spinach shoots, irrespective of the sampling date and initial bacterial inoculation doses in manure (data not shown). In experiment B, which involved the addition of the inoculation suspension both to soil and plant stem, *mapA* was detected in all 5 replicates with both *Campylobacter* strains at the day

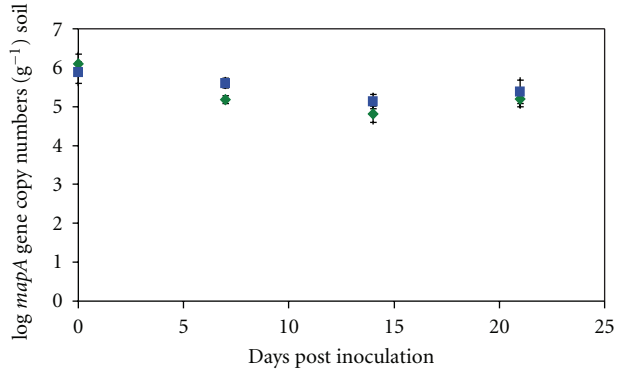

Figure 3: Comparison between the two strains of *C. jejuni* 6824 (green diamonds) and 81116 (blue squares) *mapA* gene copy numbers detected in soil in experiment B. Error bars represent standard deviations from 5 replicate samples and 3 different real-time PCR reactions per replicate.

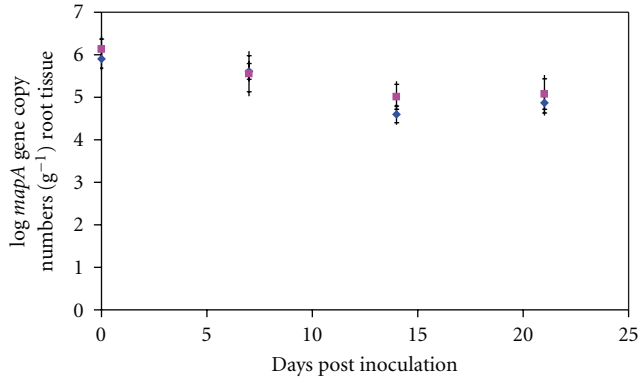

Figure 4: Comparison between the two strains of *C. jejuni* 6824 (blue diamonds) and 81116 (pink squares) *mapA* gene copy numbers detected on roots in experiment B. Error bars represent standard deviations from 5 replicate samples and 3 different real-time PCR reactions per replicate.

of inoculation (Table 1). After 7 days, the *mapA* gene was detected in 3 out of 5 replicates for strain 6824 and all 5 replicates for strain 81116. At day 14 after inoculation, *mapA* was detected in 3 out of 5 replicate shoot samples for strain 6824 and 2 out of 5 samples for strain 81116, whereas at the last day of sampling (21 days after inoculation), the gene was observed in 2 out of 5 replicates for strain 6824 and 4 out of 5 samples for 81116.

4. Discussion

Here we employ real-time PCR targeting of the *mapA* gene to evaluate the spreading pattern of *C. jejuni* strain 6824 inoculated into manure at different concentrations prior to soil application (experiment A). The same strain was used along with *C. jejuni* 81116 in a parallel experiment to establish potential differences in pathogen survival and spread between bacterial inoculation into manure and soil 14 days after planting (experiment B). As *C. jejuni*

Table 1: *Campylobacter jejuni* cells detected on spinach shoot tissue.

Day	*C. jejuni* strain 6824					*C. jejuni* strain 81116				
0	5.0	5.5	5.1	5.1	5.8	5.2	6.3	5.6	5.5	6.5
7	4.5	4.2	n.d.	n.d.	5.0	4.1	4.6	5.8	5.9	5.0
14	n.d.	n.d.	4.9	4.3	4.4	n.d.	5.3	4.4	n.d.	n.d.
21	5.5	4.4	n.d.	n.d.	n.d.	n.d.	5.3	5.5	4.6	4.9

The numbers of *Campylobacter jejuni* cells from two different strains detected on spinach shoots in five replicated pot cultures using real-time PCR. The values correspond to log *mapA* gene copy numbers g^{-1} spinach shoot tissue, and the sampling days are after *C. jejuni* inoculation. n.d.: not detected.

typically consists of a single *mapA* operon [23], the *mapA* gene copy number should be equivalent to bacterial cell number.

The use of molecular tools instead of traditional culture-based approaches has obvious advantages, including elimination of reliance on isolation and detection of viable but nonculturable cells. However, limitations, such as potential PCR inhibitors and primer specificity/sensitivity, need to be taken into account when relying on molecular techniques. The real-time PCR primers used in the present study are established as extremely sensitive. However, the detection limit observed for *C. jejuni* was still 10^4 CFU g^{-1} in soil, roots, and shoots. As *C. jejuni* was present at higher densities at most sites, this was a fairly acceptable minimum detection threshold. Possibly the most important issue involves dealing with the separation of dead and viable cells. A number of techniques to distinguish between DNA derived from dead and metabolically active cells have been established, including bromodeoxyuridine immunocapture [24, 25], use of ethidium monoazide [18, 26], and propidium monoazide [27]. However, Douglas Inglis et al. [18] showed that DNA from heat-killed *C. jejuni* cells remained in manure compost for a significantly reduced time, compared to that from viable *C. jejuni* cells, indicating that conclusions on colonization patterns may be drawn even in the absence of these activity measurement techniques. Specifically, Douglas Inglis et al. [18] demonstrated that at 64 h after inoculation of *C. jejuni*, DNA from dead cells was barely detectable, whereas that from living cells remained in the compost. Despite the physical and chemical differences between the previous and current experiments, our results consistently indicate that DNA detected and quantified in the present study is derived either from viable *C. jejuni* or possibly cells that have been dead for only a short time period. Moreover, as we saw almost no decline in bacterial cell numbers over time in the present study, the detected cells are most likely living rather than dead since the latter would have declined in number.

The initial concentrations of *C. jejuni* cells in soil were similar, regardless of whether the bacterium was inoculated directly into manure or soil 14 days later (day 0). However, at subsequent sampling events, slightly higher densities of *C. jejuni* 6824 were detected in soil amended using manure inoculated with pathogens (experiment A), compared to that to which pathogens had been added directly (experiment B). This finding suggests that bacteria adapted to conditions in manure are better equipped for life in soil, preferably in specific nutrient-rich and/or oxygen-deficient niches, signifying that manure-contaminated soil acts as a reservoir for *C. jejuni* in the field [2]. In contrast, bacterial cells added after plant emergence need to cope with other conditions, particularly a potentially more oxygen-rich aerated environment related to the development of root structure. Additionally, manure composition may play a role in the degree of *C. jejuni* persistence, as the bacteria are able to survive in stored slurries and dirty water for as long as three months, compared to a corresponding survival period in solid manure of less than one month [15]. Consequently, the slurry in the present study may have been an ideal carrier medium for *C. jejuni* applied to soil. Regardless of the initial inoculation dose, the *C. jejuni* content remained rather constant between the first and last soil sampling sessions (2–28 days), although with a slight decline. We additionally observed a significant difference in the *C. jejuni* amounts between the two strains at days 7 and 14 after planting. These strain differences may be a result of distinct genotypes, but possibly also depend on other unidentified characteristics. The fact that *C. jejuni* still were detected in soil 28 days post inoculation shows its great potential to persist in the soil environment.

The presence of *C. jejuni* on roots varied significantly between the two inoculation stages (i.e., manure inoculation prior to soil application and at 14 days after planting). Following addition to manure, no *C. jejuni* cells were detected in any of the replicates 21 days after inoculation and only sporadically in single replicates on previous sampling dates. However, in the treatment involving direct addition of *C. jejuni* to soil, positive PCR products were obtained from all replicate samples at all sampling dates for both bacterial strains, indicating that *C. jejuni* inoculated in manure adapts to the manure-soil environment before the roots develop and hence prefers the physicochemical conditions provided by organic material in soil. *C. jejuni* cells added to soil 14 days after planting possibly took advantage of root exudates and the nutrient-rich environment surrounding plant roots.

It is assumed that the majority of *C. jejuni* strains present a higher death rate than growth rate on plants [2] and better survival rate in the rhizosphere compared to the phyllosphere [2] due to the thermophilic and microaerophilic lifestyle of the bacterium. Upon addition of *C. jejuni* cells to manure, no cells could be recovered on shoots above the threshold level. However, inoculation of *C. jejuni* cells directly into the soil and on the lowest part of the plant stem led to detection of the bacterium in all replicate pots on the day of bacterial addition. This finding was probably an artefact resulting from negligent inoculation, that is, unintentional application of bacteria to the leaves. However, interestingly, the majority of *C. jejuni* cells of both strains persisted for several days on the shoots. At up to 21 days after inoculation, high levels of *C. jejuni* were detected in the majority of replicates (i.e., similar to that detected on the day of inoculation). Consequently, it appears that the probable high oxygen levels present in the phyllosphere do not significantly affect either of the *C. jejuni* strains evaluated. The bacteria may be able

to colonize specific niches on the shoots with locally low oxygen tensions, for example, within bacterial aggregates, broken tissue of plant lesions, or depressions accumulating water [2].

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