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Genetic Diversity, Antimicrobial Resistance and Survival upon Manure Storage of *Campylobacter jejuni* Isolated from Dairy Cattle Farms in the Cantabric Coast of Spain

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Simple Summary: In this study, the origin and persistence of *Campylobacter jejuni* was studied in dairy cattle farms in order to avoid recirculation of this leading cause of foodborne illness. A high level of genetic diversity and antimicrobial resistance was found, particularly in ciprofloxacin. As a result, the survival of antimicrobial resistant *C. jejuni* in cattle manure may pose a risk for human populations.

Abstract: The aim of this study was the examination of the genetic diversity and antimicrobial susceptibility of *C. jejuni* isolates obtained from dairy farms situated in Cantabria. The presence of *Campylobacter jejuni* was scrutinized in dairy farms situated in the Cantabria region (Atlantic coast, North of Spain). A total of 520 samples were collected from 12 dairy farms and 62 *C. jejuni* isolates were achieved. Sixty-one (61) of the isolates proceeded from fresh feces and only one from the stable (soil). Characterization of the isolates was conducted by Pulsed Field Gel Electrophoresis (PFGE) analysis. Antimicrobial susceptibility testing was carried out by standardized disk diffusion test. The PFGE analysis showed a high genetic diversity. From the 62 *C. jejuni* isolates, 27 different PFGE types were obtained with 70% similarity. The results of the antimicrobial susceptibility tests showed that 21 out of 27 strains were resistant to ciprofloxacin (78%), and 15 of them were also resistant to tetracycline (55%), whereas none of the 27 strains analyzed were resistant to erythromycin. *C. jejuni* was capable of surviving in livestock waste for at least 20–25 days, whereas the maximum detectable survival time on crops was of six days. This study reveals the high genetic diversity and ciprofloxacin resistance of *C. jejuni* in dairy cattle farms in Northern Spain, a fact that highlights the urgent need for the surveillance and control of this foodborne pathogen.

Keywords: thermotolerant *Campylobacter*; ciprofloxacin resistance; PFGE; risk factors; fecal samples

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1. Introduction

Campylobacteriosis caused by thermotolerant *Campylobacter* species is the most commonly reported zoonosis in the European Union (EU), with more than 240,000 confirmed cases per year [1]. These species can asymptomatically colonize a variety of wild and domestic animals [2,3] and can be transmitted to humans and lead to human infection [4]. Epidemiological studies have implicated transmission of *Campylobacter* spp. to humans by food-borne or water-borne routes, such as raw and undercooked poultry [5], raw milk [4] and tap water [6]. Campylobacteriosis is normally a self-limiting illness that does not require antimicrobial treatment. Rarely, it might lead serious sequelae and require antimicrobial therapy, especially in immunocompromised patients [7]. In such cases, the drugs of choice are macrolides (e.g., erythromycin) and fluoriquinolones (e.g., ciprofloxacin).

For that reason, the emergence of fluoroquinolone resistant *C. jejuni* poses a potential threat to these patients and the World Health Organization recently listed *C. jejuni* as one of the 12 priority pathogens due to the increase in the prevalence of fluoroquinolone resistance [8–13].

Fluoroquinolones and sulphonamides are the most commonly used families of antibiotics, and ciprofloxacin is the most widely used fluoroquinolone in the world [14,15]. Fluoroquinolone resistance in *Campylobacter* is thought to be developed due to the misuse of these compounds in both human and veterinary medicine. Antibiotics are not only prescribed for treatment, but are also administered as disease prevention measures and growth promotion, despite the ban on the use of antibiotics as growth promoters in animal feed since 2006 in the EU [16–18]. Some reports suggest that fluoroquinolone-resistant strains have a fitness advantage over wild-type *Campylobacter* [19–21]. Interestingly, it appears that fluoroquinole resistance has emerged on poultry farms even in the absence of the above-mentioned antimicrobial [22]. This led to the conclusion that other antimicrobials may select for fluoroquinolone resistance in *Campylobacter*, but the mechanisms involved are not completely clarified yet [23]. A recent study revealed that the acquisition of fluoroquinolone resistance in *C. jejuni* is associated with both the increase in viable biofilm formation under aerobic conditions as well as a more invasive phenotype in vivo and in vitro [10]. The observation of a more invasive phenotype raises the prospect that these antibiotic-resistant strains are also likely to be more pathogenic upon infection of humans.

In spite of the recognition of *C. jejuni* as an important pathogen, being one of the leading causes of gastroenteritis in humans [24], information on its epidemiology in cattle farming and the connection of its spread and persistence, through the use of manure in the field, is limited. Most manure management plans have focused on volume reduction or nutrient management with less concern for the risks associated with manure-borne bacteria. Even though only a few incidents of contamination by zoonotic agents through manure have been reported throughout the world, each one of them tended to be a serious event with human fatalities [25]. According to previous studies, the majority (65.5%) of the *Campylobacter* spp. isolated from cattle samples were identified as *C. jejuni* [26]. For that reason, the aim of this study was to determine the genetic diversity and antimicrobial susceptibility of *C. jejuni* isolates obtained from dairy farms situated in Cantabria (Spain), which is an area with a high rate of rain and high humidity. Farms using plant materials as a hot bed for livestock wastes are decreasing and the number of dunghills collecting rain water is significant, leading to high rate of slurry-based wastes. The more diluted the manure is, the less favorable conditions for composting it provides and concerns have been raised that increased slurry generation could lead to increased survival of zoonotic agents in the environment [27]. For that reason, the decline of *C. jejuni* in slurry and in pasture crops was monitored, the latter after having been spread with compost containing high concentrations of *C. jejuni*. To the best of our knowledge, this is the first study regarding the survival of *C. jejuni* in slurry and pasture crops, not only in the Cantabria region but in Spain in general and one of the very few around the world, and it is increasing our knowledge on the life cycle of *C. jejuni*.

2. Materials and Methods

2.1. Culture Media and Growth Conditions

Selective solid media for the isolation of *Campylobacter* spp. were prepared using *Campylobacter* blood-free selective agar plates (CCDA) adding CCDA selective supplement (SR0155E, Oxoid, Basingstoke, UK) to the basal agar and/or Tryptone-Soy-Blood agar (TSBA), and adding *Campylobacter* Growth Supplement (SR0232E Oxoid, Basingstoke, UK) and defibrinated horse blood (SR0050) to the basal medium, in accordance with the manufacturer's instructions (Oxoid, Basingstoke, UK). Liquid selective enrichment medium was prepared using Bolton Broth (BB), adding modified Bolton Broth selective supplement (SR0208E). All strains were maintained at −20 °C in Tryptone Soy Broth (TSB) supplemented with 20% glycerol and at −80 °C in glycerol broth (20% v/v glycerol

in 1% *w*/*v* peptone). Strains were routinely grown at 42 °C in a multi-gas incubator under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2), using anaerobic jars and CampyGenTM reagents (Oxoid, Basingstoke, UK).

2.2. Sample Collection, Processing and C. jejuni Strains Isolation

Samples were collected with sterile material, transported at 4 °C and subjected to detection of *Campylobacter* immediately upon arrival in the laboratory, usually within 2 h from the collection. All samples were enriched in 100 mL BB for 4 h/37 °C and then 48 h/42 °C, under microaerobic conditions. For solid samples, 10 g was used. For water, 3 L of sample was filtered through a 0.5 nm sterile filter which was incubated in 90 mL BB. For milk, 100 mL of sample was centrifugated at 8500 rpm/10 min and the pellet was inoculated in BB. Rectal swabs were placed directly in BB. After the enrichment step, serial dilutions up to 10^{-2} were performed in maximum recovery diluent (MRD, Oxoid, Basingstoke, UK) and the suspensions were plated on both CCDA and TSBA. Plates were incubated for 48 h at 42 °C under microaerobic conditions. All the suspected colonies were subjected to microscopic examination, Gram staining, glucose fermentation, oxidase, catalase and API test (bioMérieux, Marcy-l'Étoile, France). *Campylobacter* detection from all the samples was performed according to the method described in ISO 10272–1:2006 [28].

2.3. Molecular Methods for Species Identification and Strain Characterization

The identification and differentiation of *C. jejuni* was done by the hippuricase gene-based PCR assay [29]. Only *C. jejuni* isolates were used for further study. Characterization of the strains was conducted by PFGE following the Centers for Disease Control and Prevention PulseNet protocol for *C. jejuni* [30], using *Sma*I and *Kpn*I restriction enzymes (New England BioLabs, Ipswich, MA, USA) for cleaving the DNA. The Centers for Disease Control and Prevention standard *Salmonella* Braenderup strain H9812 was used as a reference strain during all PFGE experiments [31]. The PFGE types (or pulsotypes) were obtained by combining both restriction enzyme profiles. A PFGE profile was considered unique if one or more bands differed from other PFGE profiles [32].

This analysis of the PFGE results was performed by the use of BioNumerics software (version 4.5; Applied Maths, Kortrijk, Belgium). The similarity clustering was performed according to the instructions in the PulseNet BioNumerics manual (http://www.pulsenetinternational.org/protocols/bionumerics/, accessed on 1 January 2021). The Dice correlation coefficient was applied to identify similarities between the PFGE types with a tolerance of 1.5% and an optimization of 0.5%, generating a single dendrogram using the Unweighted-Pair Group Matching Algorithm (UPGMA).

2.4. Testing of Susceptibility to Antimicrobial Agents

The susceptibility of the organism to antimicrobial agents was determined by standardized disk diffusion tests [33]. A fixed inoculum was prepared and evenly spread on Mueller Hinton blood agar plates (bioMérieux, Marcy-l'Étoile, France) using a sterile cotton tipped applicator. After drying, antibiotic disks were placed and incubated at 37 °C for 48 h under microaerobic conditions. Erythromycin (15 µg), ciprofloxacin (5 µg) and tetracycline (30 µg) disks (bioMérieux, Marcy-l'Étoile, France) were used. Two independent determinations of the susceptibility of each *C. jejuni* strain to all three antibiotics were performed and *C. jejuni* ATCC 33291 was used as a control strain. The diameter of growth inhibition around the discs was measured and interpreted as sensitive or resistant according to the interpretive criteria provided by EUCAST [34].

2.5. Decline of C. jejuni in Manure

The experiment was repeated in two different years and seasons (October 2010 and April 2011). For each one, two independent experiments were performed and each experiment included a negative control sample (blank tank: manure not inoculated with *C. jejuni*), a positive control sample (tank with manure inoculated with *C. jejuni* strain

ATCC 33291) and samples inoculated with *C. jejuni* strain No. 427 isolated from cattle feces (this study). All *C. jejuni* strains used were grown microaerobically at 42 °C in TSB, centrifuged in order to remove media, washed and resuspended in 0.1% peptone buffer (Oxoid, Basingstoke, UK) to a density of 10^9 CFU/mL [35]. Inoculants provided 10^6 CFU/g of manure wet weight, an amount that represents a worst-case scenario in terms of pathogen loadings. Inoculated manure was mixed to give an even distribution of *Campylobacter* cells throughout.

The inoculated dairy slurry was stored in 5 m^3 above-ground circular plastic tanks. Duplicate samples were withdrawn at time zero and at intervals of 1, 2, 4, 8 days and once a week thereafter, until pathogen concentration dropped below the detection limit (100 CFU/g of manure). Each sample consisted of 10 sub-samples taken from different areas of the slurry. The presence of *C. jejuni* was monitored by survival curves generated by plate counting in CCDA [35].

2.6. Decline of C. jejuni in Pasture Crops

Liquid manure was collected in spring (April 2011), stored in 5 m^3 above-ground circular plastic tanks and left to compost for 3 months. Then, was inoculated with *C. jejuni* No. 427 as described above, and applied on crops (10% *Lolium perenne-L. roadrunner*, 30% *Festuca arundinacea schrewolfpack*, 50% *Festuca arundinacea schregreenkeeper*, 10% *Poa pratensis-L. HB129 thermal blue*), in a controlled laboratory environment (germination chamber under controlled conditions of temperature and light). Two independent experiments were performed, maintained to the average weather conditions in the region for spring and autumn time (15 °C during night and 18 °C during day; 14 h light and 10 h of darkness; 60% humidity). The amount of manure applied to the crops was calculated in order to respect the amount of nitrogen permitted according to the Directive 91/676/EEC.

The decline of *C. jejuni* on the surface of plants was detected and survival curves were generated by plate counting in CCDA [35]. Then, 10 g of pasture crops was placed in a sterile stomacher bag containing 90 mL PBS and homogenized for 1 min using a stomacher. The liquid sample was separated from the plants and all samples were enriched in 100 mL BB for 4 h/37 °C and then 48 h/42 °C, under microaerobic conditions. Serial dilutions of the mixture were used for the generation of the survival curve and the calculation of CFU/g of plant.

2.7. Analysis of Physical and Chemical Parameters of the Livestock Wastes

Parameters determined in manure were: pH, electrical conductivity, dry matter, ash, organic matter, Kjeldahl nitrogen and Olsen phosphorus, according to the instructions published by the Ministry of Agriculture of Spain [36]. Olsen phosphorus was determined according to previously described protocols [37].

3. Results

3.1. Isolation and Characterization of C. jejuni Strains

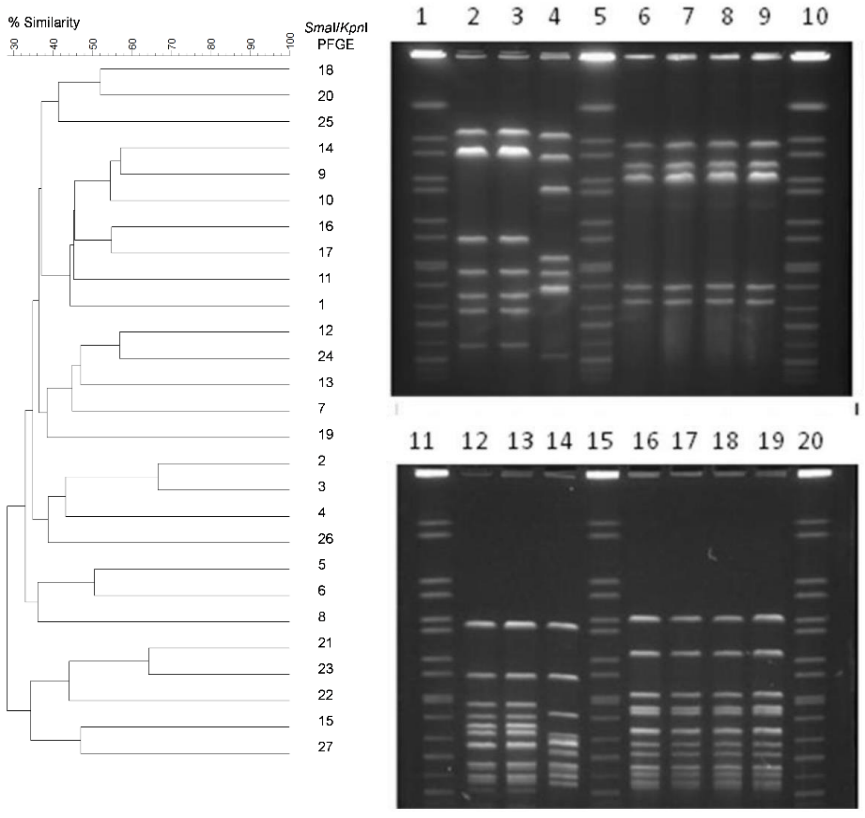
A total of 520 samples were collected and processed from years 2009 to 2012 from 12 different dairy cattle farms in Cantabria (Spain). Samples were obtained from fresh feces, stored liquid manure, water from the watering trough, filters from the milk tank and various environmental samples around the farms. Fecal samples were collected from apparently healthy dairy cows. Sixty-two (62) *C. jejuni* strains were isolated with conventional microbiological plating methods and identified by PCR, as described in Materials and Methods. Each of the 12 farms gave at least one positive result. Sixty-one (61) of the isolates proceeded from fresh feces and only one (1) from environmental samples (soil) (Table 1).

Table 1. Prevalence of *Campylobacter* isolates from different samples from dairy cattle farms in the Cantabria region (Northern Spain).

Sample Type	No of Samples Tested	No (%) of Samples Negative for *Campylobacter*	No (%) of Samples Positive for *Campylobacter*	No (%) of Samples Positive for *C. jejuni*
Raw milk	12	12 (100%)	0 (0%)	0 (0%)
Water	12	10 (83.3%)	2 (16.7%)	0 (0%)
Green grass	23	21 (72.4%)	2 (6.9%)	0 (0%)
Dry forage	11	11 (100%)	0 (0%)	0 (0%)
Maize cured forage	8	8 (100%)	0 (0%)	0 (0%)
Grass cured forage	5	5 (100%)	0 (0%)	0 (0%)
Stable floor	105	101 (94.4%)	3 (2.8%)	1 (0.9%)
Slurry tanker	47	44 (93.6%)	3 (6.4%)	0 (0%)
Fresh feces	287	129 (53.5%)	97 (40.2%)	61 (25.3%)
Stored manure	41	36 (83.7%)	5 (11.6%)	0 (0%)
Total	551	377 (72.5%)	112 (21.5%)	62 (11.9%)

3.2. Genetic Diversity of C. jejuni Isolates

The 62 *C. jejuni* isolates were characterized using PFGE in order to elucidate the genetic relationship among them. The PFGE analysis using SmaI and KpnI yielded 26 and 24 restriction profiles, respectively. The profiles obtained with the two enzymes were combined, generating 27 PFGE types or pulsotypes which were designated with numbers 1 to 27 (Figure 1a). Thus, PFGE analysis showed a high genetic diversity, as the 62 *C. jejuni* isolates were clustered into 27 PFGE types with 70% similarity (Figure 1). The PFGE types did not follow a concrete geographical pattern, as diverse pulsotypes were found in the same farm, but also strains with the same PFGE pattern were isolated from geographically distant farms (data not shown). The first strain with a unique PFGE type was considered the PFGE type strain of each PFGE type.



(**a**) (**b**)

Figure 1. (**a**) Dendrogram (UPGMA clustering based on Dice correlation coefficient) showing similarities among 62 isolates of *C. jejuni* from dairy cattle. Analysis of pulsed-field gel electrophoresis (PFGE) types

was performed as described in Materials and Methods. A total of 27 PFGE types resulted from the combination of the different PFGE restriction profiles (26 obtained with *Sma*I, and 24 with *Kpn*I). (**b**) Representative PFGE profiles obtained with *Sma*I. Lanes 2 and 3: pulsotype 1; Lane 4: pulsotype 2; Lanes 6–9: pulsotype 3; Lanes 12–14: pulsotype 4; Lanes 16–19: pulsotype 5; Lanes 1, 5, 10, 11, 15, 20: *Salmonella* serotype Braenderup reference standard (H9812) restricted with *Xba*I [31] and run under the PulseNet standardized electrophoresis conditions specific for *C. jejuni* [30].

3.3. Antimicrobial Susceptibility Testing

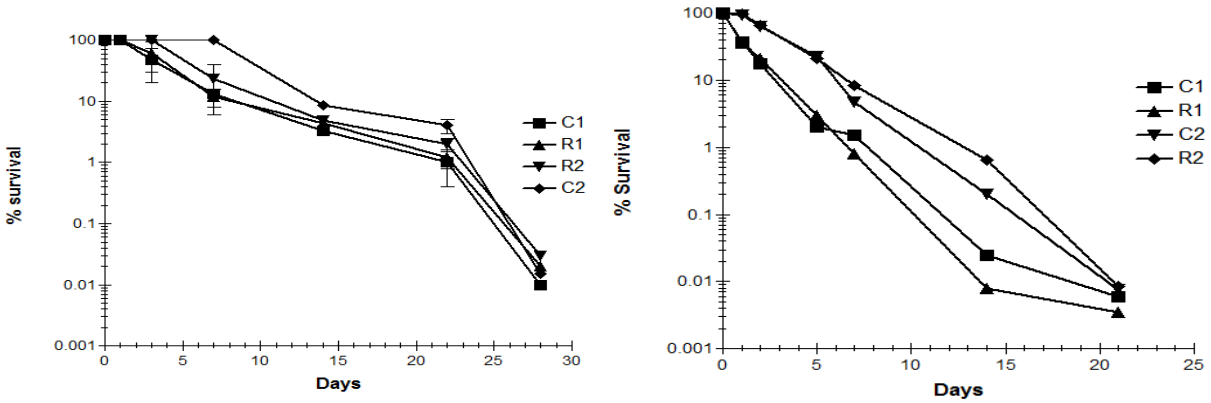
Antimicrobial susceptibility was determined for ciprofloxacin, tetracycline and erythromycin, by disk diffusion method, as described in Materials and Methods. Results are shown in Table 2. Out of 27 isolates, 21 were found to be resistant to ciprofloxacin (78%) and 15 of them were resistant to both ciprofloxacin and tetracycline (55%), whereas none was resistant to erythromycin. The control strain, which was included in all assays, was sensitive for all three antibiotics and diameters of their inhibition halos were within expected ranges.

Table 2. Antimicrobial resistance phenotypes among *C. jejuni* pulsotypes from dairy cattle samples.

Antimicrobial Resistance Phenotype	No. of PFGE Type Strains
Ciprofloxacin + tetracycline	15
Ciprofloxacin	6
Total	21

3.4. Decline of C. jejuni Strains in Livestock Waste

Two *C. jejuni* isolates were examined with respect to their ability to survive in manure. Liquid manure samples were inoculated as described in Materials and Methods and death curves were performed. After the end point of death curves, when no *C. jejuni* was detected with the plate culture media used, enrichment was performed before plating, in order to confirm the presence/absence of *C. jejuni*, which was detected for one more week. The blank tank always gave negative results for *C. jejuni*. Results are shown in Figure 2, expressed as the average ± standard deviation (n = 6). The parameters determined in the livestock wastes used are given in Table 3.



(**a**) (**b**)

Figure 2. Death curve of *C. jejuni* in livestock waste collected during autumn and inoculated with *C. jejuni* on spring (**a**) and waste collected during spring and inoculated on autumn (**b**). *C. jejuni* 427 (C1 and C2) is a strain isolated from cattle manure; *C. jejuni* ATCC 33291 (R1 and R2) is a reference strain used as a control. C1 and R1, manure sample 1; C2 and R2, manure sample 2.

Table 3. Physical and chemical parameters determined in the livestock wastes used.

Season of Collection	Type of Sample	pH	Conductivity (mS/cm)	%Dry Matter	%Ash	%Kjeldahl Nitrogen	%Ammonia Nitrogen	%Total Phosphorus
Autumn	[1] Fresh manure (1)	8.30	4.59	9.5	2.35	0.27	0.106	0.050
Autumn	[1] Fresh manure (2)	7.18	4.45	11.0	2.37	0.35	0.097	0.047
Spring	[1] Fresh manure (1)	6.84	4.16	10.3	2.15	0.30	0.081	0.070
Spring	[1] Fresh manure (2)	7.76	3.56	12.3	3.80	0.36	0.132	0.066
Spring	[2] Compost (1)	6.15	6.86	9.1	2.80	0.29	0.159	0.080
Spring	[2] Compost (2)	7.57	5.70	11.8	4.10	0.35	0.162	0.086

[1]: used to test the *C. jejuni* decline in manure. [2]: used to test the *C. jejuni* decline in crops

3.5. Decline of C. jejuni Strains upon Application of Infected Manure to Crops

Compost manure was inoculated with *C. jejuni* as described in Materials and Methods and applied on pasture crops in a controlled laboratory environment. After the end point of death curves, when no *C. jejuni* was detected with the plate culture media used, enrichment was performed before plating, in order to confirm the presence/absence of *C. jejuni*. Results are shown in Figure 3. The maximum survival time on crops was six days (four days was detected by direct plating of the samples and then two more days after enrichment of the samples).

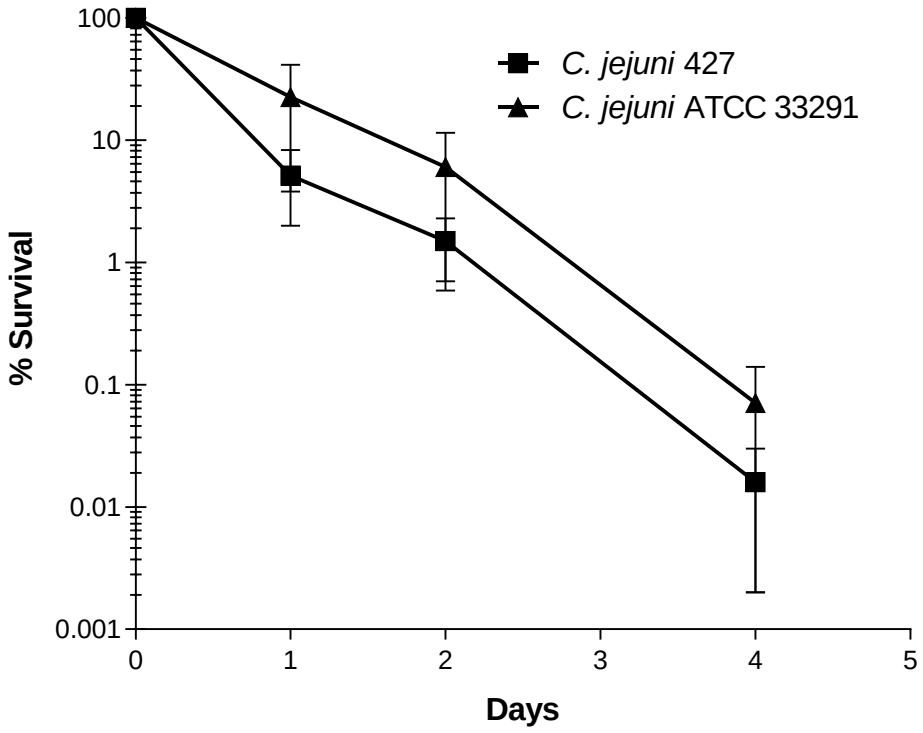


Figure 3. Death curve of *C. jejuni* in crops fertilized with infected manure. *C. jejuni* 427 is a strain isolated from cattle manure; *C. jejuni* ATCC 33291 is a reference strain used as a control.

4. Discussion

Most of the available studies are mainly concerned with the prevalence of *Campylobacter* in poultry as a main source of human *Campylobacter*iosis. However, there is concern about bovine carriage of this agent resulting in human infection [3]. In this study, 62 *C. jejuni* strains were isolated out of 520 samples from dairy cattle farms, resulting in 12% of the samples being positive to *C. jejuni*, a rate that is in accordance with other studies [11–13,38]. *C. jejuni* is often found to have lower prevalence than other zoonotic bacteria as it enters a viable but not culturable state [39,40] and maintains viability for weeks or months after cultivability loss [41]. Therefore, lack of detection of this microorganism with conventional plating methods does not necessarily mean lack of survival.

Despite the fact that most livestock manure is disposed of by application to pasture land or field crops, relatively few studies focus on the occurrence of pathogens in manure, in crops or in soils with applied manures. Studies thus far indicate that manure-borne pathogens have the ability to survive in secondary habitats (i.e., manures, soils and water) for weeks or even months, with their survival depending on the organism [42,43]. Therefore, understanding how these pathogens survive in manure and after manure application is a research priority in human safety. In this study, a *C. jejuni* isolate was examined with respect to its ability to survive in stored manure and in plants after manure application. It was found capable of surviving in livestock waste for at least 20–25 days, no matter which season of the experiment it was. The only evident difference between seasons was the time that the survivors needed to reach 0.1% of the initial population. During spring, the survivors reached 0.1% of the initial population after 15 days (Figure 2a). During autumn, the survivors reached 0.1% of the initial population after 27 days (Figure 2b). The difference of about 10 days is considered to be due to the difference of the temperature between the two seasons during the particular period of the experiment, as temperatures in autumn 2010 in Cantabria region tended to be much colder than the temperatures in spring 2011. Furthermore, it is noteworthy that during the experiment performed in autumn (Figure 2b), the death curves of the two different samples (manure 1 and manure 2) were clearly different, which is attributed to the difference of the pH between the two samples (Table 3). When manure was applied on crops, *C. jejuni* was detectable after six days (four days detectable with plating methods, six after enrichment). The experiment was carried out in a germination chamber under controlled conditions of temperature, humidity and light. It is known that survival patterns under in situ soil and field conditions are quite different from the survival studies for manure-borne pathogens conducted under controlled laboratory conditions [38,44]. Yet, it is evident that the extent of delay between manure application to fields and the introduction of cattle is an important factor determining the risk of the animals being contaminated by zoonotic pathogens present in the applied manure.

The molecular characterization of the 62 *C. jejuni* isolates by PFGE resulted in 27 PFGE types with 70% similarity (Figure 1). This result can be considered a relatively high genetic diversity for *C. jejuni* [45]. The diversity of the PFGE patterns identified in this study is consistent with previous reports in various EU countries [16,46–48], including Spain [49].

Antimicrobial susceptibility tests of *C. jejuni* pulsotypes allowed us to determine antimicrobial resistance associated with specific pulsotypes. The majority of pulsotypes (78%) were resistant to ciprofloxacin. Resistance to ciprofloxacin and tetracycline was also found between pulsotypes (55%), whereas resistance to erythromycin, usually applied for treatment of human gastroenteritis, was never encountered. Our results provide evidence that ciprofloxacin resistance in *C. jejuni* is a common phenomenon in dairy farms of Cantabria (Spain) and are in accordance with results from previous studies [11,13,50]. An increase in the number of *C. jejuni* strains resistant to frequently used antibiotics (macrolides and, especially, the quinolones) has been reported worldwide with increasing levels of resistance to ciprofloxacin and tetracycline and low resistance to erythromycin [1]. In Europe, the rates of fluoroquinolone resistance are highly variable, ranging from 1.2% in Norway to 44% in Belgium [51,52]. An alarming situation was found in Spain where the highest proportion (91.5%) of ciprofloxacin-resistant isolates was reported as well as an extremely high proportion (80.1%) of tetracycline-resistant isolates [53]. Spain has one of the highest incidences of bacterial resistance to antimicrobials, possibly linked to drug consumption patterns [54]. In such settings, the effective treatment option for human enteric *Campylobacter* infection may be significantly reduced. *Campylobacter* may induce severe or systemic infections in immunocompromised or young/elderly patients, which often requires antibiotic therapy, with the first-line antibiotics including fluoroquinolones and macrolides. Resistance to these clinically significant antibiotics, including ciprofloxacin, compromise the effectiveness of antibiotic treatments [55].

The potential of dairy cattle *C. jejuni* to resist antimicrobials, including those antibiotics of choice for treatment of human cases, reasserts the public health significance of *Campylobacter* infections in humans [56]. This study reveals that, among *C. jejuni* isolates from Spanish dairy cattle farms, resistance to ciprofloxacin occurs frequently. The reasons for the high ciprofloxacin resistance are beyond the scope of the present study. However, it is speculated that these reasons are likely to be related to the dairy farm production and management systems across different levels of the chain in the farm, as trends in antimicrobial resistance have shown a clear correlation related to the use of antibiotics in the animal production industry and antibiotic resistance [57]. On the other hand, *C. jejuni* is naturally transformable, making very likely to acquire antibiotic-resistant genes from other organisms [58]. We did not look for the mechanisms of ciprofloxacin resistance, but ciprofloxacin-resistant Gram-negative bacteria usually have *gyrA* mutations together with *parC* mutations [59]. These target site mutations can be analyzed using PCR and DNA sequencing. The results of this study indicate that the problems caused by the inappropriate use of antimicrobials extend beyond the particular food chain [60]. Due to the use of the antimicrobials in livestock, those agents appear in detectable concentrations in soil and sewage [15], a fact that suggests that liquid manure poses a risk if it is used as a fertilizer for plants that will be used to feed animals or humans. The ciprofloxacin resistance of the *C. jejuni* isolates might pose greater risks to the population; therefore, both interventions and agreements are required to implement common policies on antimicrobial usage and to minimize the emergence of *Campylobacter* resistance.

Under the "One Health" approach, which aims to sustainably balance and optimize the health of people, animals and ecosystems, it is now recognized that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent. Food safety requires high animal health standards and prevention of foodborne diseases through safety assessment of food products and establishment of practices that prevent microbial contamination. Agricultural practices, livestock production systems, animal health status, disease prevention policies, and animal management measures have an immense influence on the prevalence and occurrence of antimicrobial resistance (AMR) genes in different ecosystems and on the determination of their dynamic pathway. The prudent use of antimicrobials in Spanish dairy farms is further needed to minimize the spread of antibiotic-resistant *C. jejuni* into the environment and into the food production chain; this means minimum requirements to be followed by veterinarians when administering antibiotics to animals is an important tool to reduce the usage of antibiotics and the consecutive development of resistance [61].

Based on our results, we suggest that the presence and survival of *C. jejuni* in the farm environment could play an important role in re-infecting cattle on the same farm, making dairy cattle a potential reservoir of human *Campylobacter*iosis. One of the strategies to prevent the disease caused by *C. jejuni* is incorporation of control measures at the primary source (i.e., the animal reservoir) applying good hygiene and biosecurity measures at the farm level. However, if open pasture is used for grazing cattle, environmental control measures are more difficult to apply. Frequent contact between wildlife and farmed animals can facilitate pathogen spill over from wildlife to livestock and vice versa and eradication of *C. jejuni* can be challenging. Taking into account that *C. jejuni* could survive up to at least 6 days on crops, farmers should consider extending the time between manure application to fields and the introduction of cattle, as this could be a determining factor for the recirculation of *C. jejuni* in livestock. For dairy farms in particular, it can be difficult to prevent outbreaks and take effective precautions in advance, since the detection of *Campylobacter* is challenging. Nevertheless, prevention from fecal matter contamination, sanitizing milking equipment, avoiding contamination during repair of milking machines, and preventing silent mastitis could be the routes to prevent contamination of bulk raw milk with *C. jejuni*.

5. Conclusions

This study reveals high genetic diversity and ciprofloxacin resistance of *C. jejuni* in dairy cattle farms in Northern Spain, indicating potential risks to humans associated with dairy products or the dairy farm environment. Therefore, improvements to the environmental management of cattle farms could reduce the shedding of *C. jejuni* from cattle, thereby reducing the potential risk of *C. jejuni* at the farm level and its spread to humans through the food chain.

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Institutional Review Board Statement: No laboratory animals were used during this study. Samples were collected from 12 commercial dairy farms in the Cantabria region (Spain), after the permission of the farmers/owners and in accordance with the EU directive UE 2016/679 on "General data protection regulation". Samples collected from rectal swabs were from Holstein-Friesian dairy cows, housed free, and sampling procedures were in accordance with the EU Directive 2010/63/EU "on the protection of animals used for scientific purposes". The study was approved by the Ministry of Livestock, Fisheries and Rural Development, Government of Cantabria (Spain) and by The Official College of Veterinarians, according to the European Veterinary Code of Conduct.

Informed Consent Statement: Not applicable.

Data Availability Statement: The dataset generated during this study is available from the corresponding author upon request.

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