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Biofilm formation, virulence and antimicrobial resistance of different *Campylobacter jejuni* isolates from a poultry slaughterhouse

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ABSTRACT

The fastidious requirement of the zoonotic pathogen *Campylobacter jejuni* contrasts with its ability to overcome harsh conditions. Different strategies might be involved in the survival and persistence of *C. jejuni* through the poultry food chain. Therefore, the aims of this study were to get insights in the survival strategies in the poultry slaughterhouse environment by (i) characterizing factors such as biofilm formation, virulence and antimicrobial resistance in environmental isolates and (ii) understanding the possible link between the phenotypic and genetic characterization using whole genome sequencing (WGS). Results have shown that three STs: ST 443 (PFGE A), ST 904 (PFGE C) and ST 3769 (PFGE G), out of the six studied, formed biofilms with variable intensity according to different conditions (temperatures −37 °C, 30 °C, 25°C— and materials —stainless steel and plastic—). High levels of antimicrobial resistance were found in isolates to ciprofloxacin, nalidixic acid and tetracycline as well as to two common detergents used in the slaughterhouse. A combination of several changes in the genome of ST 904 (PFGE C) including mutations, insertions in antimicrobial resistance genes, the presence of T6SS and a set of genes related to virulence factors might explain its ability to form biofilm and persist longer in the environment. However, the complexity of the survival strategies adopted by the different strains of *C. jejuni* suggests that multiple mechanisms may exist that allow these organisms to persist and ultimately cause disease in humans.

1. Introduction

Campylobacter jejuni, a Gram negative and spiral-shaped microaerophilic bacterium, is a leading cause of human food-borne infections in developed and developing countries (Gölz et al., 2014; Lertpiriyapong et al., 2012). Although most human infections seem to be sporadic and the gastrointestinal symptoms are usually self-limiting, extra intestinal complications may develop in some cases such as reactive arthritis, Guillain-Barré and Miller-Fisher syndromes (Skarp et al., 2016). These complications are suggested to be a result of an autoimmune response induced by ganglioside-like lipooligosacharides (LOS) expressed by *C. jejuni* (Revez et al., 2011).

Poultry is the most important source of human infection reaching a colonization level in broiler ceca as high as 10^9 CFU/g (Ma et al., 2014; Marotta et al., 2015). These high counts allow the bacteria to be easily spread throughout the processing plant (Whiley et al., 2013) and contaminate carcasses by spilled gut content during slaughter. Although *Campylobacter* has uniquely fastidious growth requirements and it is very sensitive to environmental conditions, the bacteria are able to overcome harsh conditions such as cleaning and disinfection procedures (Peyrat et al., 2008), persist for longer periods in the slaughterhouse environment (García-Sánchez et al., 2017) or survive throughout the poultry food chain (Melero et al., 2012).

Biofilm formation is considered an important strategy to allow bacteria to survive in suboptimal conditions in the environment. However, molecular mechanisms involved in regulating biofilm formation of *C. jejuni* are still poorly understood. To date a number of characterized virulence genes may also be involved in the development of biofilms. For instance, genes responsible for cell motility (*flaA, flaB, flaC, flaG, fliA, fliS* and *flhA),* cell surface modifications (*peb4*, *pgp1* and *waaF),* quorum sensing (*luxS*) and stress response (*ppk1*, *spoT*, *cj1556*, *csrA*, *cosR*, *cprS* and *nuoK).* CosR is likely to be a key protein in the maturation of *C. jejuni* biofilm and is also involved in the expression of the antimicrobial efflux pump CmeABC (Turonova et al., 2015). This efflux pump plays a key role in *C. jejuni* physiology, conferring intrinsic and acquired resistance to diverse toxic compounds, such as bile salts,

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antibiotics or various detergents (Lin et al., 2002, 2003).

Despite the importance of *C. jejuni* as an enteric pathogen and the progress in recent years in comprehending the complicated and multifactorial pathogenesis, there is a gap in understanding the combination of phenotypic and genotypic characteristics. The challenge now is to link the genotypic and phenotypic data to better understand the mechanisms influencing *C. jejuni* persistence in the environment, and the role that this might play in transmission of this pathogen (Bronowski et al., 2014). Therefore, the aims of the study were (i) the phenotypic characterization of different strains isolated from a poultry slaughterhouse to get insights into biofilm formation, virulence profile and antimicrobial resistance and (ii) understanding the possible relationship between phenotypic characteristics and genotypic profile using whole genome sequencing (WGS).

2. Material and methods

2.1. Bacterial isolates

In this study, forty-five *Campylobacter jejuni* strains were used. Those strains were isolated in a previous study from a Spanish slaughterhouse environment, clustered in six PFGE profiles and after whole genome sequencing, assigned to six MLST sequence types (ST) and clonal complexes (CC) (García-Sánchez et al., 2017). Moreover, in that study, strains were sequenced proportionally to the number of isolates obtained according to the sampling procedure: 33 strains corresponded to pulsotype G (ST 3769; ST 21 CC), 8 to pulsotype C (ST 904; ST 607 CC) and 1 from the rest of pulsotypes A (ST 443; ST 443 CC), B (ST 775; ST 52 CC), D (ST 464; ST 464 CC) and F (ST1074; ST 460 CC). Table 1 shows the epidemiological data related with those STs obtained from PubMLST (https://pubmlst.org/campylobacter/).

2.2. Biofilm formation

The ability of all *C. jejuni* strains to form biofilm was determined on two different materials: stainless steel and 96-well polystyrene microtiter plates (Nunclon Delta Surface, Thermo Scientific). Moreover, three different temperatures 37 °C, 30 °C and 25 °C were tested under aerobic and microaerobic conditions. Sterile stainless steel coupons (stainless steel type 1.4301 according to European Standard EN 10088-1, with a type 2B finish according to European Standard EN 10088-2) were placed in a six-well polystyrene tissue culture plate and incubated with 3 mL of Nutrient broth (Oxoid, Basingstoke, England). Likewise, 96 well-polystyrene microtiter plates were incubated with 300 µL of Nutrient broth (Oxoid). Each *C. jejuni* strain was cultured and aliquots of Nutrient broth with an absorbance value (A600) of 0.05 (~10^7 cfu/mL) were added to each well. After 48 h of incubation, in the different conditions, surfaces were washed with an equal volume of phosphate-buffered saline and dried at 60 °C for 30 min. Next, 1 mL of 1% (wt/vol) crystal violet solution was added, and further incubated on a shaker at room temperature for 30 min. The unbound dye was removed by thorough washing in water, followed by drying at 37 °C. Bound crystal violet was dissolved by adding 20% acetone/80% ethanol, followed by incubation on a shaking platform for 15 min at room temperature (Brown et al., 2014). The resulting dissolved dye was measured at a wavelength of 590 nm in an Epoch spectrophotometer (BioTek, Winooski, USA) by using the Gen5 2.00 software. *C. jejuni* reference strain NCTC 11168 was used as positive control (Kalmokoff et al., 2006; Reuter et al., 2010; Turonova et al., 2015). All assays were performed in triplicate.

2.3. Antimicrobial, detergent and disinfectant susceptibility testing

The Minimum Inhibitory Concentration (MIC) for a range of antimicrobial compounds, antibiotics, industrial detergents and disinfectants, against all *C. jejuni* strains was determined by agar dilution method and performed in triplicate (CLSI, 2013). Each bacterial inoculum was diluted in 2 mL of 0.9% NaCl and the turbidity was adjusted to 0.5 McFarland units. The different strains were spotted into Nutrient agar (Oxoid) supplemented with 5% sheep blood agar (Oxoid) containing the respective antimicrobial.

Six antibiotics belonging to four different classes were tested. Two fluoro(quinolone): ciprofloxacin (0.03–64 mg/L) and nalidixic acid (4–128 mg/L); two macrolides: erythromycin (0.12–16 mg/L) and azithromycin (0.015–1 mg/L); one aminoglycoside: gentamicin (0.12–8 mg/L); and tetracycline (0.12–128 mg/L). Isolates were considered to be susceptible or resistant based on epidemiological cutoff values according to the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org). Multidrug resistance (MDR) was defined as simultaneous resistance to three or more unrelated antimicrobials (Mäesaar et al., 2016). *C. jejuni* ATCC 33560™ was used as a positive control strain.

In addition, six commercial sanitizers, commonly used in the cleaning and disinfection of poultry processing plants were tested. From these, four were detergents: Desenfort (Betelgeux, Gandia, Spain), Freefoam (Diversey, Barcelona, Spain), Deltafoam (Diversey, Barcelona, Spain) and Diverflow (Diversey, Barcelona, Spain), named C_1, C_2, C_3, C_4, respectively, in order to simplify the terms. The chemical composition of these was: a mix of sodium and potassium hydroxide (C_1), mix of organic acids (C_2), alkaline detergent with silicates (C_3) and sulfamic acid (C_4). The remaining two compounds were quaternary ammonium disinfectants commercially available: Dexacide (Betelgeux, Gandia, Spain) and Divosan (Diversey, Barcelona, Spain), named D_1 and D_2, respectively. The tested concentrations in the study included, at least 10 dilutions, decreasing or increasing 0.25% (vol/vol), depending on the first results obtained in the test performed in the laboratory and starting in the lower concentration recommended by suppliers for each sanitizer.

2.4. Analysis of genes associated to antibiotic resistance and virulence

All isolates were subjected to analysis for the presence of antimicrobial resistance genes using ResFinder v2.1 (Zankari et al., 2012) available at the CGE (https://cge.cbs.dtu.dk/services/ResFinder) (Table 2).

Additionally, the genomes of all *C. jejuni* strains were analysed for the presence of 68 virulence associated genes selected according to previous published works (Chen et al., 2005; Dasti et al., 2010;

Table 1
Epidemiological data of the different pulsotypes used in this study.

PFGE/MLST	Records PubMLST	Origin	Distribution	Presence in Spain	Year[a]
A/ST 443; ST 443 CC	59	Human, Chicken	World	Madrid	2010
B/ST 775; ST 52 CC	89	Human, Chicken	Europe	Yes, Not specified	2002
C/ST 904; ST 607 CC	104	Human, Chicken	Europe	Vitoria, Madrid	2003/2010
D/ST 464; ST 464 CC	613	Human, Chicken	World	Castile and Leon, Madrid	2008/2010
F/ST 1074; ST 460 CC	7	Human, Chicken	UK/Spain	Vitoria	2002–2004
G/ST 3769; ST 21 CC	14	Human, Chicken	UK, Luxembourg, Belgium, Portugal	No	

[a] Year that the Sequence Type appeared in Spain according to PubMLST (https://pubmlst.org/campylobacter/) Feb 2019.

Table 2
Antibiotic resistance mechanisms found in *Campylobacter jejuni*.

Antibiotic classes	Encoding gene(s)	Resistance mechanism	References
Quinolones, (fluoro)quinolones	*gryA*	Modification of the DNA gyrase target (Thr-86-Ile; Asp-90-Asn, Ala-70-Thr)	Hakanen et al. (2002)
		Efflux through CmeABC	Cagliero et al. (2006a)
			Iovine (2013)
Tetracycline	*tet(O)*	Modifications of the target ribosomal A site by TetO binding	Iovine (2013)
		Efflux through CmeABC and possibly others	Lin et al. (2002)
Macrolides	23rRNA	Mutations in 23rRNA.Mutations in ribosomal proteins L4/L22 is likely minor	Cagliero et al. (2006b)
		Efflux though CmeABC and possibly others	Lin et al. (2007)
		Decreased membrane permeability due to MOMP	Iovine (2013)
Aminoglycoside	*aphA*, *aadE*	Modification of the antibiotic by modifying enzymes	Iovine (2013)
		Contribution of efflux is not clear	
Beta-Lactam	*bla*OX-*61*	Inactivation of the antibiotic by enzyme B-lactamase	Griggs et al. (2009)
	*bla*OX-*184*	Efflux through CmeABC and possibly others	Iovine (2013)
		Decreased membrane permeability due to MOMP	

Table 3
Campylobacter jejuni virulence genes selected in this study.

Response to stress	*ppk1, ppk2, cstA, spoT, hspR, htrA, htrB, sodB, katA, perR, ahpC, dnaJ, cosR, cprR, cprS, nuoK*
Motility/Chemotaxis	*flaA, flaB, flaC, fliA, rpoN, luxS, cheA, cheY*
Adhesion	*cadF, capA, peb4, pgp1, waaF, ilvE, csrA, MOMP/porA, peb1A, flpA*
Invasion	*ciaB, ciaC, ciaD, ciaI, iam, ceuE, virB11*[a]
Capsule	*kpsM, kpsE, pgld*
LOS	*cst-II*[a], *cst-III*
Toxins	*cdtA, cdtB, cdtC, cgtB*[b], *wlaN*
Iron up take	*fur, cfrA, cfrB*[a]
Colonization	*ggt*[a]
T6SS[c]	*tssA, tssB, tssC, tssD, tssE, tssF, tssG, tssH, tssI, tssJ, tssK, tssL, tssM*

All sequences correspond to reference strain NCTC 11168, except for.
[a] *Campylobacter jejuni* 81–176 strain.
[b] *Campylobacter jejuni* OH4384 strain.
[c] *Campylobacter jejuni* 414 strain.

Bronowski et al., 2014; Bolton, 2015). Among the selected genes are those involved in stress response, motility and chemotaxis, adhesion, invasion, capsule, LOS class, toxins, iron uptake system, colonization and the gene *wlaN* related to Guillain-Barré syndrome (Table 3). Additionally, genes related to the Type VI (T6SS) secretion system involved in pathogenesis and symbiotic relationship/adaptation to environment were also studied.

Assembled contigs obtained from a previous work (García-Sánchez et al., 2017) were annotated using PROKKA v1.12 (Seemann, 2014), and further used by Roary v3.12.0 (Page et al., 2015) to produce the *C. jejuni* pangenome to analyse the presence of the 68 virulence genes in each strain. BLASTN (https://blast.ncbi.nlm.nih.gov) was used for those virulence genes not present in the reference strain *C. jejuni* NCTC 11168 (*virB11*, *cgtB*, *cst-II*, *ggt* and *cfrB*) and T6SS. The sequence of these genes was found on GenBank (https://www.ncbi.nlm.nih.gov/genbank). Coverage of 95% and identity of 90% of any of the sequences in the alignments were required.

2.5. Statistical analysis

All individual results were recorded using MS EXCEL software version 15.41 (Microsoft Corporation; Redmond, WA, USA) and statistical analyses were performed with the software program Statgraphics Centurion XVI. At least three biological replicates (each with three technical replicates) were used to calculate means and the standard errors of the mean. The effect of two variables atmosphere with two levels (aerobic and microaerobic) and temperature with three levels (37 °C, 30 °C and 25 °C) on the ability of *C. jejuni* to form biofilms was determined by multifactor analysis of variance (multifactor ANOVA). Main effect of atmosphere was not statistically significant ($p < 0.05$). On the contrary, main effect of temperature was significant ($p < 0.05$). The ability of the different PFGE/ST CC of *C. jejuni* to form biofilms compared to positive and negative controls were statistically analysed using one-way analysis of variance (ANOVA). A Fisher LSD (Least Significant Difference) test was applied to determine group differences at 95% significance level.

3. Results

3.1. Biofilm formation

The intensity of biofilm formation of the different MLST profiles in comparison with the positive control strain *C. jejuni* NCTC 11168 is shown in Table 4. Production of biofilms under microaerobic and aerobic conditions did not differ significantly ($p < 0.05$). Results show that ST 904; ST 607 CC (PFGE C) formed biofilm at 37 °C, 30 °C and 25 °C, and in both materials (polystyrene and stainless steel), with increased biofilms production at 37 °C than at the lower temperatures. ST 3769; ST 21 CC (PFGE G) formed biofilms also in both materials and atmospheres. However, biofilms were more labile and had a clear dependence on the temperature (Table 4). In both cases, no statistical differences were observed within the different strains belonging to the same MLST profile. Moreover, ST 443; ST 443 CC (PFGE A) only formed biofilms on stainless steel coupons. In contrast, the other genotypes ST 775; ST 52 CC (PFGE B), ST 464; 464 CC (PFGE D) and ST 1074; ST 460 CC (PFGE F) did not form biofilms under the conditions investigated.

Table 4
Results of biofilm formation by different *C. jejuni* types on stainless steel and plastic surfaces compared to negative and positive controls.

C. jejuni	Stainless steel			Plastic		
PFGE/MLST	37 °C	30 °C	25 °C	37 °C	30 °C	25 °C
A/ST 443; ST 443 CC	-	++	+	-	-	-
B/ST 775; ST 52 CC	-	-	-	-	-	-
C/ST 904; ST 607 CC	+++	+	+	+++	++	+
D/ST 464; ST 464 CC	-	-	-	-	-	-
F/ST 1074; ST 460 CC	-	-	-	-	-	-
G/ST 3769; ST 21 CC	-	+	-	+	+	-

(−) = No biofilm formation; no significant difference from negative control ($p < 0.05$).
(+) = Weak biofilm formation; significantly higher than negative control and lower than positive control ($p < 0.05$).
(++) = Moderate biofilm formation; no significant difference from positive control ($p < 0.05$).
(+++) = Strong biofilm formation; significantly higher than positive control ($p < 0.05$).

Table 5
Antimicrobial susceptibility (MIC) and their relationship with resistance genes in *C. jejuni* Sequence Types.

C. jejuni	Quinolones			Tetracycline		Macrolides				Aminoglycoside		B-Lactam	Efflux Pump	
PFGE/MLST	NAL	CIP	R[a] T86I/others	TC	R[a] *tet (O)*	ERY	AZT	R[a]	R L22 Mut/Ins	GM	R[a]	R[a]	CmeABC	CmeR (Mutation)
A ST 443; ST 443 CC	R (128) [b]	R (8)	T86I S22G, N203S, R285K	R (128)	+	S (0.25)	S (0.03)	–	A103V, S109A, V137A	S (0.25)	–	*blaOXA-184*	+	G144D, S207G
B ST 775; ST 52 CC	R (128)	R (32)	T86I S22G, N203S, R285K	R (128)	+	S (0.5)	S (0.06)	–	A103V, S109A, V137A	S (0.5)	–	*blaOXA-61*	+	I100V, A108T, V109I, I115V, G144D, N150D, E189K, S207D
C ST 904; ST 607 CC	R (128)	R (32)	T86I N203S, R285K	R (128)	+	S (2)	S (0.06)	–	I65V, S109A, V137A K118_T119 insAPAAKK	S (0.5)	–	*blaOXA-184*	+	G144D, E189K, S207D
D ST 464; ST 464 CC	R (128)	R (8)	T86I R285K	R (128)	+	S (1)	S (0.06)	–	A103V, S109A, V137A	S (0.5)	–	*blaOXA-61*	+	T6I, G144D, P183R, S207G
F ST 1074; ST 460 CC	R (128)	R (8)	T86I S22G, N203S, A206V, R285K	R (128)	+	S (0.12)	S (0.0015)	–	I65V, A103V, S109A, V137A	S (0.5)	–	-	+	G144D, S207G
G ST 3769; ST 21 CC	R (128)	R (8)	T86I N203S, A206T, R285K	R (16)	+	S (1)	S (0.06)	–	A103V, S109A, V137A	S (0.5)	–	*blaOXA-61*	+	No mutations found

NAL, nalidixic acid; CIP, ciprofloxacin; TC, tetracycline; ERY, erythromycin; AZT, azithromycin; GM, gentamicin; B-Lactam, beta-lactam.

[a] R: resistance mechanism. R T86I/others: point mutations in the subunit A of the DNA gyrase gene; R L22 Mut/Ins: point mutations and insertion in 23S ribosomal protein L22; R: presence of B-Lactam *blaOX-61/184* gene.

[b] Interpretation of MIC for *C. jejuni* epidemiological cutoff values: NAL (≥16 mg/L), CIP (≥0.5 mg/L), TC (≥1 mg/L), ERY (≥4 mg/L), AZT (≥0.25 mg/L), Gm (≥2 mg/L); R = resistance; S = sensitive.

3.2. Antimicrobial, cleaner and disinfectants susceptibility

As shown in Table 5, resistance to 2 classes of antimicrobials was observed in 45 of the isolates examined. All the isolates were resistant to the (fluoro)quinolones (nalidixic acid and ciprofloxacin) and tetracycline. Although no differences were observed in the MIC values within the same MLST profiles, some differences were observed among the different MLST genotypes.

Table 5 also shows the relationship between the phenotypic characteristics of isolates within the different MLST profiles and the specific antimicrobial resistance genes detected by ResFinder. All isolates resistant to the fluoro(quinolones) had a mutation in T86I of DNA gyrase conferring this resistance. Similarly, all the genotypes resistant to tetracycline carried the *tet(O)* gene. Additionally, ResFinder found that the majority of MLST profiles had the *bla*OX resistant gene to beta-lactam in their genome. The gene *bla*OXA-184 was present in profiles ST 904 (PFGE C) and ST 443 (PFGE A) and *bla*OXA-61 in ST 3769 (PFGE G), ST 775 (PFGE B) and ST 464 (PFGE D). In contrast, ST 460 (PFGE F) did not possess resistance genes to this class of antimicrobial. Although all isolates were resistant to macrolides, most of the STs harbored point mutations in 23S ribosomal protein L22 and in the case of ST 904 (PFGE C) a six amino acid insertion. Moreover, all STs harbored genes encoding the efflux pump CmeABC associated with multidrug resistance. Differences among the six STs related to the mutations in the gene encoding the regulatory protein, CmeR, were observed ranging from no mutations in ST 3769 (PFGE G) to eight mutations in ST 775 (PFGE B) (Table 5).

Detergent and disinfectant susceptibility analyses have shown that two detergents and two disinfectants (C_1, C_2, D_1 and D_2) were effective against all *C. jejuni* strains tested according to concentrations recommended by suppliers (Table 6). However, two detergents (C_3 and C_4) were not effective at the lower recommended concentration by suppliers. All tested strains from the different STs were resistant to C_3 at 3% (vol/vol), the lower concentration; but sensitive from 3.25% (vol/vol). Furthermore, C_4 was the least effective against all strains tested, showing resistance at twice the concentration of the lower limit recommended by the supplier (Table 6).

3.3. Genes associated with virulence factors

All STs studied were positive for almost all of the 68 virulence-associated genes, with few exceptions. Only STs 904 (PFGE C), 464 (PFGE D) and 1074 (PFGE F) harbored all genes related with the presence of T6SS. Table 7 shows the differences among genotypes.

4. Discussion

In this study, 45 *C. jejuni* strains belonging to 6 different STs, isolated in a previous work (García-Sánchez et al., 2017), were characterized phenotypically and genotypically in order to get insights in their survival and persistence in the slaughterhouse environment. Other authors have showed that some *C. jejuni* strains from abattoir environments may be less sensitive to stresses and therefore, reach the final products (Kudirkienè et al., 2011). Moreover, not all *C. jejuni* strains appear to have the same virulence, survival and host adaptation potential and certain metabolic activities appear to be strain dependent (Revez et al., 2011). As a result, different strategies may be employed by cells to overcome the stresses that could prevail within the processing plant environment. Among these strategies, it is well known that biofilm formation on contact surfaces contributes to the persistence of foodborne pathogens along the food chain.

Results showed that from the six STs tested in this study only three: ST 443 (PFGE A), ST 904 (PFGE C) and ST 3769 (PFGE G), were able to form biofilms. These three STs agree with the results obtained in a previous work where they were isolated after 4, 21 and 17 days, respectively, from a slaughterhouse environment, including dirty and clean surfaces and broiler meat (García-Sánchez et al., 2017). Of these, ST 904 (PFGE C) was able to form biofilm with a higher intensity than the others and at different temperatures and air conditions. It must be considered that in the plucking and evisceration steps there are high temperatures and humidity (12-22 °C and 90–100% RH), together with equipment design that may facilitate biofilm formation. The lower temperature range cited in this study refers to air, suggesting that the actual temperature on equipment surfaces should be higher as the water temperature in scald tanks is around 53 °C. Moreover, some authors have mentioned that chicken juice allows increased attachment, providing a conditioned surface for the bacteria to adhere to and prolonging the viability of *C. jejuni* and other bacteria (Brown et al., 2014; Birk et al., 2004). In all STs studied, the addition of chicken juice to *in vitro* experiments increased biofilm production (data not shown). Additionally, several authors have described the presence of *C. jejuni* after cleaning and disinfection in the slaughter environment (Peyrat et al., 2008) being a source of broiler carcass contamination during the slaughter process (Kudirkienè et al., 2011; Melero et al., 2012).

The presence and expression of antimicrobial resistance mechanisms might also favor the development of biofilms and the persistence of *C. jejuni* strains after cleaning the processing surfaces, especially at subletal concentrations. In this study, all tested strains showed resistance to fluoro(quinolones) and tetracycline, whereas all strains were sensitive to the macrolides, erythromycin and azithromycin and to the aminoglycoside, gentamicin. Resistances to fluoro(quinolones) were related to the presence of the T86I substitution in the GyrA protein in all strains tested, which has been shown to confer high-level resistance (Zhang et al., 2017). Moreover, ResFinder identified a number of unusual substitutions in this protein in the different STs. Among them, substitution R285K was found in all STs, and substitution N203S in five of them. However, these substitutions do not affect the quinolone resistance determining region (QRDR) of the gene *gyrA* (codons 69 to 120) (Hakanen et al., 2002), and it appears they do not have any effect on the MIC in the different STs characterized in the current study.

The *cmeABC* operon that encodes a multidrug efflux pump, which plays a key role in bacterial physiology, has been associated with intrinsic and acquired resistance to antimicrobial compounds and is required for bacteria to adapt to environmental stresses (Lin et al., 2002; Routh et al., 2009; Zhang et al., 2017). Transcription of this operon is repressed by protein CmeR. The gene (*cme*R) encoding this protein is located immediately upstream of the cmeABC operon and its expression blocks the transcription of the *cme*ABC operon. Mutations in the promoter region of this operon or in CmeR result in an overexpression of the efflux pump. According to ResFinder, all STs harbored several substitutions in the regulatory protein CmeR, with the exception of ST 3769 (PFGE G). Among them, substitutions E189K and S207D in the C-terminal region only appeared in ST 775 (PFGE B) and ST 904 (PFGE C). Interestingly, these STs showed a higher MIC (32 mg/mL) in

Table 6
Percentage (% vol/vol) of detergents and disinfectants tested in *C. jejuni*.

PFGE/MLST	Detergent				Disinfectant	
	C_1	C_2	C_3	C_4	D_1	D_2
	1–3%	2–10%	3–10%	0.5–3%	0.5–3%	1–2%
A/ST 443; ST 443 CC	0.25[a]	0.25	3	1	0.0015	0.0125
B/ST 775; ST 52 CC	0.25	0.25	3	1	0.062	0.0215
C/ST 904; ST 607 CC	0.25	0.25	3	1	0.062	0.062
D/ST 464; ST 464 CC	0.25	0.25	3	1	0.031	0.062
F/ST 1074; ST 460 CC	0.25	0.25	3	1	0.031	0.062
G/ST 3769; ST 21 CC	0.25	0.25	3[b]	1	0.031	0.062

[a] Concentrations represent the limit of growth. Concentrations above these values inhibited growth of *C. jejuni* strains.

[b] Three strains belonging to this PFGE type were resistant to concentrations of 2.75 (% vol/vol) or above.

Table 7
Differences in virulence gene profiles among the *C. jejuni* strains investigated.

PFGE/MLST	Adhesion		Toxins		LOS class		Colonization	*T6SS*
	capA	*virB11*	*cgtB*	*wlaN*	*cst-II*	*cst-III*	*ggt*	
A/ST 443; ST 443 CC	–	–	–	–	–	–	–	–
B/ST 775; ST 52 CC	+	–	–	–	–	–	–	–
C/ST 904; ST 607 CC	+	–	–	–	+	–	–	+
D/ST 464; ST 464 CC	–	–	–	–	+	–	+	+
F/ST 1074; ST 460 CC	+	–	+	–	+	–	–	+
G/ST 3769; ST 21 CC	+	–	–	+	–	+	–	–

comparison with the other three STs (8 mg/mL). According to several authors, amino acid substitutions in CmeR do not affect its binding ability to the *cmeABC* promoter, but a mutation that led to C-terminal truncation of CmeR prevented its DNA-binding activity (Grinnage-Pulley and Zhang, 2015). Although these substitutions are not a truncation, the charge balance of the C-terminal region changes. However, from data obtained in this study it is not possible to check the effect of those changes in CmeR performance. In addition, CmeR functions as a pleiotropic regulator and modulates the expression of at least 28 other genes in *C. jejuni*, apart from repressing the transcription of *cmeABC*.

The different STs were also sensitive to macrolides erythromycin and azithromycin. However, ST 904 (PFGE C) again showed higher resistance to those antibiotics (2 mg/mL) than the other STs. Some substitutions found present in that ST, such as I65V, A103V, and S109A, have been previously identified in erythromycin-susceptible and resistant *C. jejuni* isolates (Wei and Kang, 2018). These authors also reported an insertion of six amino acids between 114 and 115 positions in three *C. jejuni* strains showing azithromycin resistance and reduced susceptibility to erythromycin. A similar insertion of six amino acids 'APAAKK' between the K118 and T119 positions in L22 was found in ST 904 that might have some influence in the higher resistance observed compared to the other STs evaluated. Further studies should be done to investigate this issue.

Presence of organic matter in slaughterhouse surfaces, observed after cleaning and disinfection procedures, might cause a decrease in the effectiveness of the disinfectant used. In this situation, cleaning personnel used to increase the application dose of chemical sanitizers in order to improve cleaning efficiency. However, some authors have suggested that the increase in disinfectant doses which may impose a selective pressure and contribute to the emergence of disinfectant resistant microorganisms (Langsrud et al., 2003; Peyrat et al., 2008). Moreover, some disinfectants are not fully biodegradable and may persist in sewage for long periods. For instance, quaternary ammonium compounds will only degrade under aerobic conditions, resulting in continuously fluctuating concentration gradients that might promote the development of resistance in bacteria (Tezel and Pavlostathis, 2015). In this study, ST 904 (PFGE C) demonstrated the highest level of resistance to antimicrobials and was also found to form the strongest biofilm. This ST also showed the highest level of resistance to quaternary ammonium disinfectants (0.062 in D_1 and D_2).

Therefore, this study suggests that there might be a relationship between strains that displayed both high levels of antimicrobial resistance and biofilm formation. Biofilms can facilitate the exchange of genetic material among bacteria. Efimochkina et al. (2018) have described this phenomenon after culturing *C. jejuni* in subinhibitory antibiotic doses. Although the current study only evaluated biofilm formation by single species of bacteria, it is likely that complex biofilms develop *in vivo* involving multiple species (Brown et al., 2014; Teh et al., 2016) or into preexisting biofilms such as the ones formed by *Pseudomonas aeruginosa* (Culotti and Packman, 2015). Henceforth, these options cannot be ruled out in the case of those STs that showed weak or moderate biofilm formation ability *in vitro*.

Virulence factors may also contribute to the survival of some genotypes in harsh environments. However, there is a gap in understanding the relationship between presence/absence of genes and their expression and ability to form biofilms. All STs possessed 48 of the 68 virulence genes selected and none harbored the *virB11* gene. Some differences between STs appeared for the 19 remaining genes, 6 virulence genes and the 13 genes corresponding to T6SS. Protein CapA plays a role in host association and colonization by *Campylobacter* and mutants for this protein failed to colonize and persist in chickens (Ashgar et al., 2007). This gen appeared in two STs that form biofilms (PFGE C and PFGE G). The gene *wlaN* was detected in ST 3769 (PFGE G) and according to some authors it is associated with Guillain-Barré syndrome (Datta et al., 2003; Koolman et al., 2015). This pulsotype harbored the gene *cst-III* related to LOS class C. Finally, the set of genes encoding T6SS was present in ST 904 (PFGE C), but not in the other two biofilm formers ST 443 (PFGE A) and ST 3769 (PFGE G). T6SS is implicated in adaptation, quorum and stress sensing, bacterial growth and motility, biofilm formation and destruction of competing bacteria (Lertpiriyapong et al., 2012; Ugarte-Ruiz et al., 2014). All pulsotypes where T6SS was present had the gen *cst-II* (LOS class A/B).

In summary, this article reflects the complexity of relating phenotypic features with genetic information in the different STs characterized. According to the results, it appears that survival in harsh conditions may be dependent on the expression of several genes within STs. It is suggested that adaptation to environmental stresses results from a range of genetic alterations. In that sense, ST 904; ST 607 CC (PFGE C), isolated during 21 days in a previous work has showed the highest biofilm production and antimicrobial resistance. Moreover, genetic information showed that it harbors all genes related with T6SS, an insertion of 6 amino acids in the L22 RNA protein, two amino acid substitutions in the C-terminal of CmeR protein and 63 genes related with virulence factors. Other STs in this study revealed different genetic characteristics and did not form extensive biofilms. However, further studies will be needed in order to better understand the ecological and genetic characteristics of the different *C. jejuni* STs and prevent their persistence along the poultry food chain, decrease the antimicrobial resistance and human risk exposure.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.05.016.

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