Assessment of biofilm formation by *Campylobacter* spp. isolates mimicking poultry slaughterhouse conditions

P. M. Araújo, E. Batista, M. H. Fernandes, M. J. Fernandes, L. T. Gama, and M. J. Fraqueza[1]

CIISA – Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon 1300-477, Portugal

ABSTRACT This research aimed to assess the biofilm formation ability of *Campylobacter* strains under temperature and oxygen stress conditions, similar to those found in the industrial environment, to explain the persistence of this pathogen on the poultry slaughter line. A collection of *C. jejuni* and *C. coli* isolates (n = 143) obtained from poultry samples (cecal content and neck skin), collected at slaughterhouse level, from diverse flocks, on different working days, was genotyped by *flaA*-restriction fragment length polymorphism (**RFLP**) typing method. A clustering analysis resulted in the assignment of 10 main clusters, from which 15 strains with different *flaA*-RFLP genotypes were selected for the assessment of biofilm formation ability and antimicrobial susceptibility. Biofilm assays, performed by crystal violet staining method, were conducted with the goal of mimicking some conditions present at the slaughterhouse environment, based on temperature, atmosphere, and contamination levels. Results indicated that many *C. jejuni* strains with similar *flaA*-RFLP profiles were present at the slaughterhouse on different processing days. All the strains tested (n = 15) were multidrug-resistant except for one. Biofilm formation ability was strain-dependent, and it appeared to have been affected by inoculum concentration, temperature, and tolerance to oxygen levels. At 10°C, adherence levels were significantly lower than at 42°C. Under microaerobic and aerobic atmospheres, at 42°C, 3 strains (*C. jejuni* 46E, *C. jejuni* 61C, and *C. coli* 65B) stood out, exhibiting significant levels of biofilm formation. *C. jejuni* strains 46E and 61C were inserted in clusters with evidence of persistence at the slaughterhouse for a long period of time. This study demonstrated that *Campylobacter* strains from broilers are capable of forming biofilms under conditions resembling the slaughterhouse environment. These results should be seen as a cue to improve the programs of hygiene implemented, particularly in those zones that can promote biofilm formation.

Key words: *Campylobacter*, poultry, slaughterhouse, biofilm, food safety, *flaA*-RFLP

2022 Poultry Science 101:101586
https://doi.org/10.1016/j.psj.2021.101586

INTRODUCTION

Campylobacter has been reported as the most common bacterial pathogen to cause human gastroenteritis in the EU since 2005. *Campylobacter jejuni* species, in particular, is accountable for the vast majority of cases, followed by *Campylobacter coli* (EFSA, 2021).

Poultry reservoir is generally the primary source of infection (Teh et al., 2014; Skarp et al., 2016), which emphasizes the importance of *C. jejuni* and *C. coli* survival and persistence at the slaughterhouse environment, the last stage of the poultry meat production chain, before selling at retail. Effects of adverse environmental conditions on the vital functions of bacterial cells lead to stress responses associated with changes in gene expression. Bacteria can survive in biofilms, transitioning to a "dormant state", while preserving metabolic activity and suspending cell division. Bacterial communities living in a biofilm possess extracellular polymeric substances (**EPS**), forming a thick matrix that prevents the diffusion of chemicals inside the biofilm. Consequently, these bacteria are more tolerant to various stresses, both chemical and physical. Biofilms can occur on surface materials and food surfaces, and therefore they represent a food safety concern and a central subject to study (Efimochkina et al., 2017).

The persistence of *C. jejuni* and *C. coli* in the slaughter line results from cross-contamination of poultry carcasses during specific processing steps, namely those related to the poultry scald tank (scalding), the feather plucking machine, vent cutting, and other evisceration equipment (Corry et al., 2017). The dismantlement of some types of equipment can be complex, and joints,

Received July 7, 2021.
Accepted November 2, 2021.

[1]Corresponding author: mjoaofraqueza@fmv.ulisboa.pt

corners, cracks, crevices, and valves, are known to be vulnerable points for biofilm formation (Rossi et al., 2017). In this way, even the best cleaning and sanitizing programs cannot compensate for faulty equipment or inaccessible areas (Rossi et al., 2017). Examples of sites prone to biofilm formation by pathogenic bacteria, according to some studies, are the rubber fingers of feather plucking machines, plastic curtains, conveyor belt materials, stainless steel bolts and screws (which are difficult to dismantle and clean), and wastewater collection systems (gutters, drains) (Carpentier and Cerf, 1993; Dhakal et al., 2019). Furthermore, the slaughter line environment crosses different zones, from dirty zones to clean zones, with different temperatures and humidity levels. All these factors seem to explain *Campylobacter*'s ubiquity along the slaughterhouse line, contributing to the challenge of controlling this pathogen, despite its fastidious particularities. *Campylobacter* requires a reduced oxygen atmosphere (microaerobic) and an optimal growth temperature of 42°C. Additionally, it is susceptible to several environmental stresses found in the food industry, described by several authors, such as temperature, low pH, oxygen, and osmotic stress (Solomon and Hoover, 1999; Kim et al., 2015).

C. jejuni was reported as capable of forming biofilms on food industry-related surfaces such as stainless steel (Sanders et al., 2007; Brown et al., 2014; Teh et al., 2016, Wagle et al., 2019). However, the influence of the surface material is not the only factor that matters, since the presence of fecal content, organic detritus, other microorganisms, and even chicken exudate likely aids biofilm formation (Zhong et al., 2020). Chicken juice (a food-based model) enhances biofilm formation, and *C. jejuni* bacteria preferentially bind to chicken juice particles rather than directly to the abiotic surface (Brown et al., 2014).

Campylobacter spp. is more fastidious when compared to other foodborne pathogens, such as *Salmonella enterica* and enterohemorrhagic *Escherichia coli*. Besides having more restricted growth requirements, the bacteria lacks several stress tolerance genes commonly found in other enteric pathogenic bacteria (Oh et al., 2019). From this perspective, *C. jejuni* theoretically should not survive and persist along the poultry chain. This paradox has been the focus of many studies suggesting that biofilm formation could be the underlying mechanism that allows the pathogen to survive and spread (Teh et al., 2014, Oh et al., 2019).

Even though it was reported that *C. jejuni* can form biofilms, it is arguable if strains from poultry sources/slaughterhouse environments can form biofilms since most of the conducted studies have used reference strains and ideal growth conditions (Lamas et al., 2018). Although a reasonable number of studies is already available (Efimochkina et al., 2017; Melo et al., 2017; Farfán et al., 2019; García-Sánchez et al., 2019), there are few studies about *Campylobacter* biofilm formation capacity under stress conditions that mimic those found in the food industry (Teh et al., 2014).

In this context, a representative collection of *Campylobacter* isolates (n = 143) was gathered, obtained from different poultry flocks at the slaughterhouse level. This work aimed to screen the ability of different *C. jejuni* and *C. coli* strains to form biofilms and survive under different conditions mimicking the slaughterhouse environment; the selection of strains was based on the different *flaA*-RFLP genotypes obtained and antimicrobial susceptibility profiles.

MATERIALS AND METHODS

Sampling and Isolation of Campylobacter *spp*

The sampling was performed from November 2018 to June 2019. At the slaughterhouse level, samples of broiler carcasses, neck skin, and cecal content were collected from diverse flocks on different working days. For neck skin, sampling was performed according to the recommendations described in the regulation (EU) 2017/1495 of August 23, 2017. *Campylobacter* enumeration and isolation were performed according to ISO 10272-1 and 10272-2 (2017). Fecal samples were obtained from cecum extracted from broiler carcasses as Fraqueza et al. (2016) described. Fecal samples were streaked with a loop on mCCD agar (*Campylobacter* selective agar, Neogen) supplemented with Cefoperazone and Amphotericin (Neogen). All plates were incubated at 42°C for 48 h under microaerobic conditions: 6% O_2; 7.1% CO_2; 7.1% H_2 (Anoxomat, Advanced Instruments). All isolates presenting typical colony morphology were submitted to the Gram staining procedure and oxidase, catalase, and Hippurate test, as described in ISO 10272-1 (2017). *Campylobacter* isolates were stored in cryotubes with Brain Heart Infusion broth (Scharlau, Spain) and 15% glycerol at −80°C. For DNA extraction, the culture from each isolate was consistently stored in Eppendorf tubes with TE 1X buffer (10 $mmol^{-1}$ Tris-HCl, 1 $mmol^{-1}$ EDTA, pH = 8). A total of 143 *C. jejuni* and *C. coli* isolates comprised this collection.

Reference Strains

The reference strain *C. jejuni* NCTC11168 was kindly provided by Naoaki Misawa from the University of Miyazaki, Miyazaki, Japan. *C. coli* SVA was kindly provided by Eva Olsson Engvall from Sweden CRL/SVA collection, European Community Reference Laboratory (**CRL**), Uppsala, Sweden. *Campylobacter jejuni* ATCC 33560 (CCM 6214) was kindly provided by Mónica Oleastro from INSA (National Institute of Health Dr. Ricardo Jorge).

DNA Extraction

DNA extraction was carried out by the Chelex100 extraction method. The main steps for DNA extraction

included vortexing, centrifugation, pellet resuspension in 6% Chelex100 (Merck, Germany) solution, boiling, cooling, and final centrifugation (12,000 rpm for 5 min). Nonpolar DNA was retained in the supernatant above the Chelex (Lienhard and Schäffer, 2019).

The DNA of each isolate was stored in a 1.5 mL Eppendorf tube at −80°C, until usage. The NanoDrop device (Thermoscientific) was used to check the amount and purity of extracted DNA.

Multiplex PCR for cdtABC

A multiplex PCR was performed to detect *cdtA, cdtB*, and *cdtC* genes, as described by Samosornsuk et al. (2007), using VWR Dopio (VRW, Belgium) thermocycler, for the confirmation of isolates as *C. jejuni* or *C. coli*.

Extracted DNA from the reference strains *C. jejuni* NCTC 11168 and *C. coli* CRL/SVA was used for positive controls. As a negative control, a "blank" tube was made, with no DNA extract and solely DNAse free water (Sigma life science, UK) for every PCR reaction performed. Gel Red (nucleic acid staining solution, Biotium, Fremont, EUA) and bromophenol blue (Merck, Germany) were homogenized with PCR products before gel loading. PCR products were run in a 1.5% agarose gel (SeaKem LE Agarose, Lonza), with TBE (Tris-borate-EDTA) 1X buffer, for 45 min under 90 V. DNA molecular ladder used ranged from 100 bp to 1,000 bp (NZYTech ladder V). PCR products were visualized and photographed under UV light with ChemiDoc XRS + (Biorad Laboratories).

Genotyping of Campylobacter Isolates

PCR Amplification of flaA Gene The flagellin gene locus of *C. jejuni* contains 2 flagellin genes (*flaA* and *flaB*), which are arranged in tandem, and separated by approximately 170 nucleotides. The *flaA* gene is highly conserved and possesses variable regions, making this locus suitable for restriction fragment length polymorphism (**RFLP**) (Wassenaar and Newell, 2000). The polymerase chain reaction was performed to amplify 1,713 bp *flaA* gene, according to Wassenaar and Newell (2000). The cycling conditions submitted in thermocycler VWR Dopio (VRW, Belgium) were as follows: initial denaturation step at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing 50°C for 1 min, extension at 72°C for 1 min; and final elongation step at 72°C for 5 min. For positive control, DNA extracted from *C. jejuni* NCTC11168 and *C. coli* CRL/SVA was used. PCR products were submitted to agarose gel electrophoresis. Gel Red (nucleic acid staining solution, Biotium, Fremont, EUA) and Bromophenol blue (Merck, Germany) loading dye were homogenized with PCR products. PCR products (1,713 bp) were run in a 1.5% agarose gel (SeaKem LE Agarose, Lonza), with TBE (Tris-borate-EDTA) 1X buffer, for 45 min under 100V. The DNA molecular weight marker used ranged from 200 to 10,000 bp (NZYTech ladder III). PCR products were visualized and photographed under UV light with ChemiDoc XRS+MP Imaging System (Biorad Laboratories).

RFLP The *flaA* PCR products (1,713 bp) were digested with *DdeI* restriction enzyme (New England Biolabs, UK) for one hour at 37°C, according to the manufacturer's instructions for master mix preparation. Bands separation was performed by gel electrophoresis on 2% agarose gel, with TBE 1X (Tris-Borate-EDTA) buffer. The molecular weight marker ranged from 100 bp to 1,000 bp (NZYTech, ladder V). Gel red was the nucleic acid stain used for bands visualization under UV light (ChemiDoc XRS+, Biorad Laboratories). Photographs of the *flaA*-RFLP band patterns were taken and saved throughout the experiment.

Isolates Subculture

Strains stored in cryotubes at −80°C in BHI (Brain Heart Infusion broth, Scharlau, Spain) with 15% glycerol were reactivated by subculturing for 48 h in Bolton broth (Oxoid Ltd. UK) supplemented with 5% sheep defibrinated blood (Thermoscientific) at 42°C, in microaerobic conditions (Anoxomat, Advanced Instruments). After recovery, a loop (10 μL) was taken from cultures in Bolton broth and inoculated in Columbia agar plates with 5% sheep blood agar (COS) (bioMérieux, France), incubated at 42°C during 48 h, under microaerobic conditions.

Antibiotic Susceptibility Profile

Antibiotic susceptibility assessment was performed for ten antibiotics by the standard disc diffusion method, according to EUCAST (2020) and Fraqueza et al. (2016). Isolates were recovered, and bacteria cells were removed from COS agar plates (bioMérieux) to prepare suspensions adjusted to 0.5 MacFarland. Each suspension was inoculated with a swab in Mueller-Hinton agar plates supplemented with 5% sheep blood (bioMérieux). Plates were incubated at 42°C for 48 h in a microaerobic atmosphere. The tested antibiotics were: ampicillin (10 μg), amoxicillin + clavulanic acid (20 + 10μg), tetracycline (30 μg), erythromycin (15 μg), gentamicin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), nalidixic acid (30μg), trimethoprim/sulfamethoxazole (25 μg) and ertapenem (10 μg) (Oxoid Ltd.. The diameter of the inhibition zones was measured with calipers and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) guidelines, and the Comité de l'antibiogramme de la Société Française de Microbiologie (2020).

A zone diameter breakpoint of ≤19 mm was defined as resistant for the specific case of ertapenem, according to CLSI guidelines (2018), concerning zone diameter breakpoints for *Enterobacteriaceae*.

Assessment of Biofilm Formation, Mimicking Slaughterhouse Conditions

The biofilm formation assay was designed to mimic industry conditions by considering temperature, atmosphere, nutrients available, and contamination levels. The poultry slaughter line crosses different ambient temperatures ranging from 30°C (in dirty areas) to 10°C to 12°C (in clean areas), and 0 to 4°C in the chilling and storage areas (where carcasses are rapidly refrigerated and kept at 0−4°C). Since the slaughter process involves different ambient temperatures, the assessment of biofilm formation was performed at 10°C and 42°C, under aerobic and microaerobic atmospheres. Furthermore, the culture medium was supplemented with 10% chicken juice once it aids biofilm formation (Brown et al., 2014). Chicken juice also provides raw chicken nutrients, which mimic available nutrients in the processing environment (Birk et al., 2004). Lastly, regarding the contamination levels expected to be at slaughterhouse equipment and carcasses, 2 different inoculum concentrations were investigated in this study in two separated assays. The first assay (assay 1) tested a low inoculum concentration, corresponding to 10^3 CFU/mL. The second assay (assay 2), tested a higher concentration of inoculum, correspondent to 10^6 CFU/mL. These inoculum concentrations were chosen because 10^3 CFU/mL is the limit established in the Regulation (EU) 2017/1495 for broiler carcasses. However, *Campylobacter* contamination levels can be much higher than 10^3 CFU/g on equipment and carcasses contaminated with fecal content. Perez-Arnedo and Gonzalez-Fandos (2019) reported that in the intestinal tract of broilers, values range between 5 and 9 log CFU/g. Given that leakage of the intestinal tract sometimes occurs during evisceration and in the dirty areas of the process line, it was essential to assess biofilm formation at a higher dose of inoculum (10^6 CFU/mL).

Chicken Juice Preparation The chicken juice was prepared according to Pang and Yuk method (Pang and Yuk, 2018), with slight modifications. Briefly, a fresh chicken (weight of ± 1.3 kg) was placed inside a sterile vacuum bag with 700 mL of sterile saline solution. The carcass juice was collected and centrifuged at 12,000g for 15 min to remove solids. Afterward filter sterilization with 0.22 μm pore-size syringe filters (Membrane Solutions) was performed. Sterilized chicken juice was stored at −20°C. When thawed for usage, it was filtered again as described.

Quantitative Biofilm Formation Assay Overnight cultures (20 mL) of *Campylobacter* strains selected (n = 15) were made with Nutrient Broth No.2 (Oxoid) supplemented with 10% chicken juice for assays 1 and 2. The absorbance (A) (Pharmacia Ultrospec 2000 UV/VIS Spectrophotometer, UK) of overnight cultures was adjusted with culture medium to reach the desired inoculum concentration for each assay. Plate counts were done in Campyfood agar plates (bioMérieux) to establish and confirm the relation between A600 nm and CFU/mL.

In assay 1, the dilution 10^{-2} of culture with A600 nm set approx.= 0.40−0.44 was used for a cell suspension to reach 10^3 CFU/mL. In assay 2, to reach 10^6 CFU/mL in cell suspension, A600 nm was set approx. = 0.50−0.55. Aliquots of 200 μL were taken from the prepared cell suspensions and placed in the individual wells of 96-well polystyrene plates (Frilabo, Portugal). Each strain was inoculated in 3 consecutive lanes (3 × 6) in the 96-well plates. For the positive control strain, *C. jejuni* NCTC 11168, two repetitions (two lanes 2 × 6) were inoculated in every plate performed (2 × 6). For the negative control, two lanes were filled only with culture medium in every plate as well. For both assays, plates were incubated for 72 h, under 4 different conditions: a) 42°C microaerobic; b) 42°C aerobic; c) 10°C microaerobic; and d) 10°C aerobic.

The crystal violet staining method was performed as described by O'Toole (2011) for biofilm quantification. Briefly, culture media was removed, and wells were washed three times with distilled water by manual pipetting. Total biomass was measured after fixation with 1% crystal violet (Sigma Life Science, India) solution, followed by 3 washing steps. The last washing step was made by submerging the plates in a small container with distilled water to guarantee the adequate cleaning of crystal violet residuals. Elution of crystal violet was performed with alcohol/acetone solution (80%/20%). Eluted dye was removed from each well and placed in a new 96-well microtiter plate for reading at OD580 nm (absorbance microplate reader, Tecan Sunrise, Switzerland). After 72 h of incubation, readings were performed.

Data Analysis

Bionumerics software version 6.6. (Applied Maths, Belgium) was used to analyze all *flaA*-RFLP profiles obtained for each isolate (n = 143). Dice coefficient and UPGMA clustering method (unweighted pair group method with arithmetic mean) were used for clustering analysis, with an optimization setting of 1.5%, and a band position tolerance of 1.5%.

A binary simple matching and UPGMA clustering method were performed to create a dendrogram for antimicrobial susceptibility data. Lastly, the cluster analysis was also performed for the selected isolates with Dice coefficient and unweighted pair group method using arithmetic averages (**UPGMA**) of both experiences (*flaA*-RFLP and antibiotic susceptibility test).

For biofilm assay data, the Statistical Analysis Systems (**SAS**) software package, version 9.4 (SAS Institute, Cary, NC), was used to conduct a statistical analysis. The GLM procedure of SAS was used to perform an analysis of variance. The main factors considered were: strains, temperature, atmosphere, and inoculum concentration. The model also considered interactions between strains, temperature, and atmosphere. The OD580 nm values obtained from the crystal violet staining method were corrected for all isolates by subtracting the mean of negative controls (wells inoculated only with culture medium) in each plate.

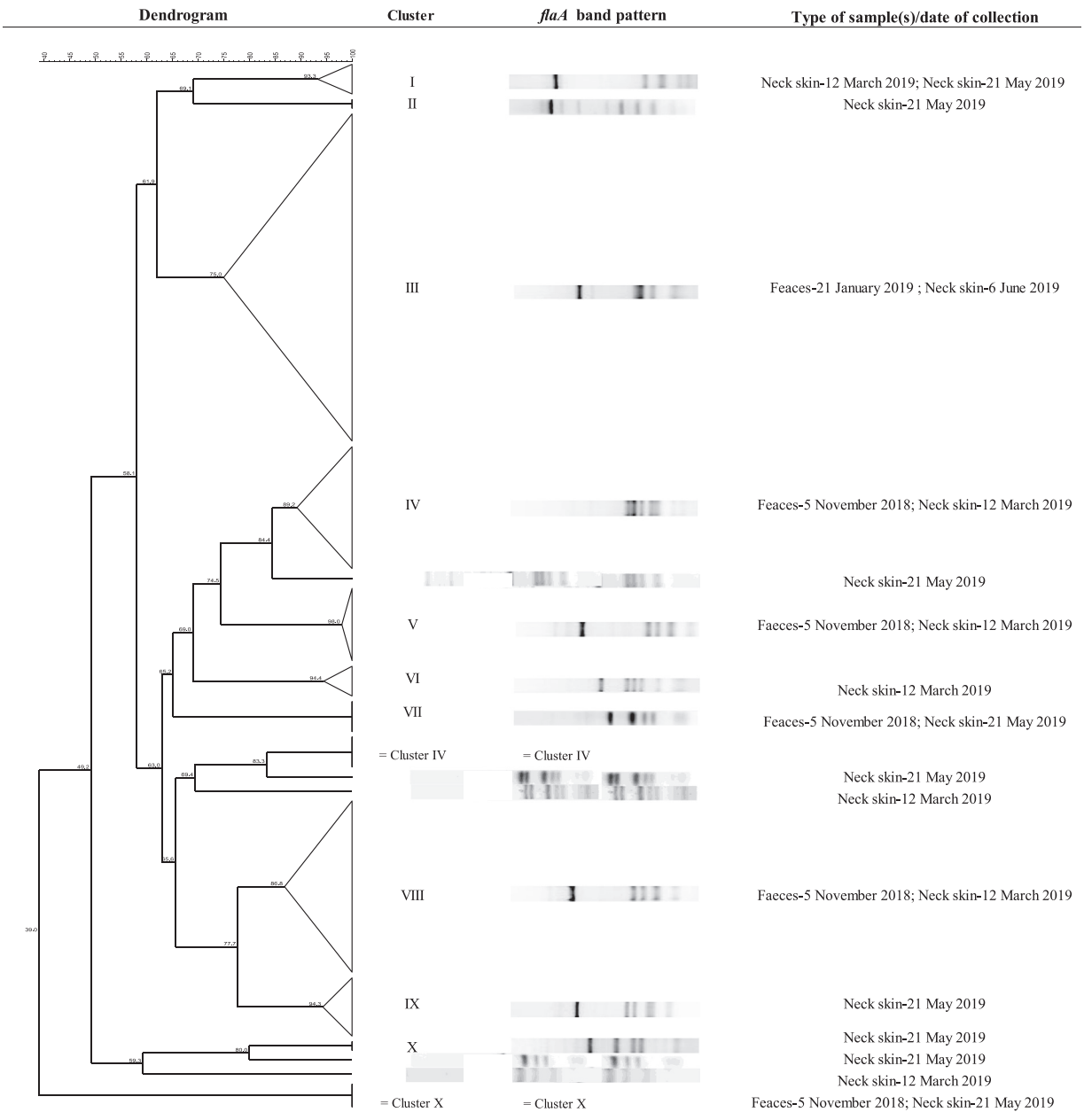

Figure 1. Dendrogram showing the assigned clusters for *Campylobacter* strains selection.

RESULTS AND DISCUSSION

Genotyping Characterization of Campylobacter Isolates

All the isolates (n = 143) presented *cdtABC* genes needed to produce the cytolethal distending toxin (**CDT**).

Clustering analysis performed with Bionumerics allowed the assignment of twelve main clusters presented in Figure 1, where all *fla*A-RFLP profiles (n = 143) were grouped. However, after detailed observations, 10 clusters were ultimately assigned due to slight variations in gel runs that resulted in lower similarity percentages for identical band patterns. For this reason, clusters III, VIII, IV, and X were formed with a similarity below 90%. One strain from each cluster was randomly selected, along with 5 strains with unique profiles (total of 15 strains with different *flaA*-RFLP profiles), for antimicrobial susceptibility test and biofilm formation assays.

Two significant observations can be made from the dendrogram formed, that is, large genetic variability and persistence of strains over a long period. Considerable genetic variability was found for the sampling day 21 March 2019, comprising 7 different genotypes obtained. Also, neck skin samples collected on November 5, 2018 resulted in 5 different genotypes. The genetic variability found in *Campylobacter* isolates was previously reported in *C. jejuni* and *C. coli* isolates in other studies using various typing methods (Abd Damjanova et al., 2011; Wieczorek et al., 2015; Zbrun et al., 2017; El-Hamid et al., 2019). According to Vidal et al. (2016), commercial broiler farms provide

Table 1. Resistance profiles of 15 *Campylobacter* strains tested (*C. jejuni* = 13; *C. coli* = 2).

Strain	Specie	Resistance profiles
106E	*C. jejuni*	AMP-ERI-TET-CIP-NA-SXT-ETP*
63E	*C. jejuni*	AMP-ERI-TET-CIP-NA-ETP*
105E	*C. coli*	AMP-ERI-TET-CIP-NA-AMC
104B	*C. jejuni*	AMP-ERI-TET-CIP-NA-SXT
64D	*C. jejuni*	AMP-ERI-TET-CIP-NA
106B	*C. jejuni*	
22A	*C. jejuni*	AMP-TET-CIP-NA-SXT
20C	*C. jejuni*	
65B	*C. coli*	
105-1BR	*C. jejuni*	AMP-TET-CIP-NA
61C	*C. jejuni*	
106A	*C. jejuni*	
105B	*C. jejuni*	
46E	*C. jejuni*	AMP-CIP-NA-SXT
65E	*C. jejuni*	AMP-CIP-NA

AMP = Ampicillin (10 μg); ERI = Erythromicin (15 μg); TET = Tetracycline (30 μg); CHL = chloramphenicol (30 μg); GEN = Gentamicin (10 μg); CIP = Ciprofloxacin (5 μg); NA = Nalidixic acid (30 μg); AMC = Amoxacillin + Clavulanic acid (20 + 10 μg); STX = Trimethoprim + Sulfamethoxazole (25 μg); ETP = Ertapenem (10 μg).

*Resistance to Ertapenem is presumptive.

"an ecological niche for a wide variety of genotypes," causing an impact on the structure of *Campylobacter* populations found in broiler production.

Besides the diversity observed, the persistence of genotypes throughout the period studied was assessed by analyzing the sampling days inside each cluster. The most common band pattern obtained was the one recognized in cluster III. There was evidence of persistence within this cluster (the biggest one formed) from January 2019 to June 2019. For the second-largest cluster (VIII), results show an identical genotype in isolates from fecal samples collected in November 2018 and a neck skin sample collected in March 2019. This result indicates the persistence of the genotype found in this cluster (VIII) from November 2018 to March 2019. Other clusters that indicated persistence during this period were clusters IV and V. In addition, in clusters VII and X, the samples collected in November 2018 also had a genotype identical to that presented by isolates obtained in samples collected in May 2019. In this way, several clusters presented the persistence of some genotypes during the period studied.

Another exciting finding was observed in Cluster IX, which comprised only samples collected on the same day yet correspondent to 4 different broiler producers. Because it is unlikely that the same genotype would correspond to different poultry farmers, this cluster may indicate cross-contamination occurring at the slaughterhouse.

Antibiotic Resistance Patterns of *Campylobacter* Strains

Antibiotic resistance profiles obtained for all *Campylobacter* strains (n = 15) are found in Table 1. Also, a dendrogram (Figure 2) was created, to associate *flaA* genotypes with the resistance profile of each strain. All strains were resistant to ampicillin (**AMP**), nalidixic acid (**NA**), and ciprofloxacin (**CIP**). On the contrary, all strains were susceptible to chloramphenicol and gentamicin. The observed levels of resistance towards AMP and NA were expected (100%), due to the known widespread resistance to these antibiotics in Portugal

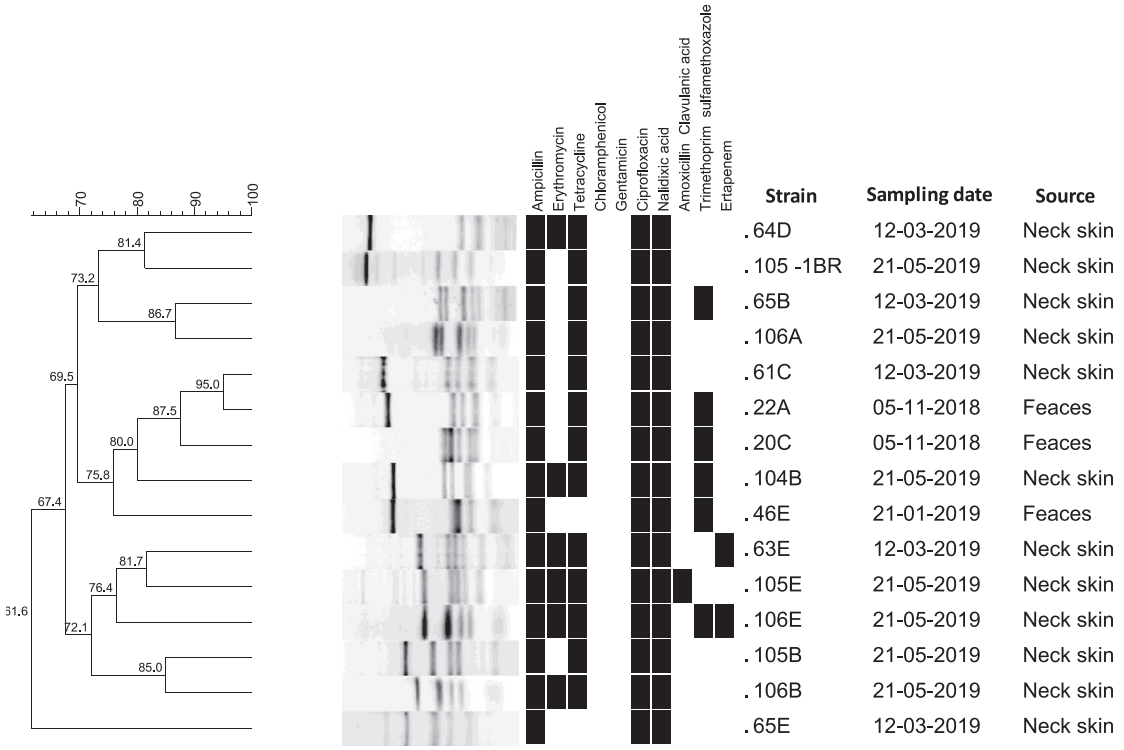

Figure 2. *Campylobacter flaA*-RFLP profiles of the 15 strains selected and their cluster association according to profile similarity and antibiotic resistance profile. Black squares represent resistance and white represents sensitivity.

(Duarte et al. 2013, Fraqueza et al., 2016). The high susceptibility to chloramphenicol (**CHL**) and gentamicin (**GEN**) was also expected since resistance to these antibiotics is reported to be very rare (Whitehouse et al., 2018).

EFSA (2020) indicated that Europe, in general, has reported that the highest resistance rates in isolates recovered from poultry meat were found for CIP, NA, and tetracycline (**TET**) (overall percentages ranging from 54 to 83%). Our results are in line with those obtained for other European countries since the highest resistance rates were also found for NA (100%), CIP (100%), and TET (88%). Duarte et al. (2013), also reported high resistance rates for NA (100%), CIP (92.8%), and TET (76%) in 125 clinical strains of *C. jejuni* and *C. coli* collected during the period of 2009 −2012, in Portugal.

Following tetracyclines, the highest resistance levels in our study were obtained for erythromycin (**ERI**) and trimethoprim-sulfamethoxazole (**SXT**). The ERI resistance was found to be particularly concerning since this antibiotic is a treatment option for persistent *Campylobacter* infections (Kayman et al., 2019). The occurrence of *Campylobacter* isolates with combined resistance to erythromycin and ciprofloxacin are of major concern to public health since both of these antibiotics are recognized as 'critically important antimicrobials' for the treatment of persistent *Campylobacter* infections in humans (EFSA, 2020). In this study, 6 out of 15 strains had combined resistance to CIP and ERI, that is, *C. jejuni* 106E, *C. jejuni* 63E, *C. coli* 105E, *C. jejuni* 104B, *C. jejuni* 64D and *C. jejuni* 106B. This result was in agreement with the frequency of macrolide resistance (35%) reported by Fraqueza et al. (2014) in 82 *C. jejuni* isolates from poultry origin.

Two *C. jejuni* strains had a potential resistance to ertapenem, specifically *C. jejuni* 106E and *C. jejuni* 63E. MIC should be assessed, for example with E-test gradient MIC strips to confirm this resistance. Also, Carbapenems resistance is sporadic in *C. jejuni* and *C. coli*, and is not even well defined by advisory boards such as EUCAST. Furthermore, carbapenem antibiotics are not licensed in food-producing animals in the EU, North America, and Australia. In all circumstances, EFSA recommends that the use of such antibiotics in animal production should be actively discouraged globally (EFSA, 2013), but rare cases of resistance occurring from the treatment of persistent infections have been emerging. For example, a recent clinical case was reported in Japan after long-term oral antibiotic treatment with tebipenem and faropenem for a persistent infection caused by *C. coli* (Hagiya et al., 2018).

Regarding the dendrogram in Figure 2, it shows different *flaA* profiles linked to similar antibiotic resistance profiles. Still, in the case of *C. jejuni* 61C and 22A, these strains were confirmed as different, due to their different resistance profiles, despite their very similar *flaA*-RFLP genotype.

The most common resistance profile was: AMP-TET-CIP-NA found in four strains (Table 1): *C. jejuni*105-1BR, *C. jejuni* 61C, *C. jejuni* 106A, and *C. jejuni* 105B. The *C. jejuni* strain 61C was in the second largest cluster obtained in the dendrogram for all *flaA*-RFLP band profiles (n = 143). This observation indicates that a significant proportion of isolates obtained in the poultry slaughterhouse likely have combined resistance to TET and CIP. The two strains resistant to the lowest number of antibiotics tested were *C. jejuni* 46E (AMP-CIP-NA-SXT) and *C. jejuni* 65E (AMP-CIP-NA). The strain *C. jejuni* 46E represents the largest cluster formed (i.e., the most common genotype found), reinforcing that probably most isolates obtained in slaughterhouses are multidrug-resistant.

The strain *C. jejuni* 106E, with the highest number of acquired resistances, was inserted in a small cluster. In this cluster, most isolates were collected in May 2019, but there was one isolate from feces collected in November 2018 with the same genotype. This result indicates that probably clones from *C. jejuni* strain 106E *flaA* genotype persisted in the slaughterhouse from November 2018 to May 2019.

Furthermore, in the 15 strains tested, the occurrence of multidrug resistance (resistant to at least one agent in three or more antimicrobial categories) was high, such that all strains were multidrug-resistant except one (*C. jejuni* 65E). A high frequency (69%) of multidrug resistance was also reported by Fraqueza et al. (2016) in isolates obtained from quails (in a total of 91 isolates, *C. coli* n = 87; and *C. jejuni* n = 4).

Campylobacter Biofilm Formation Mimicking Slaughterhouse Conditions

The behavior of different strains of *Campylobacter* was tested according to three main factors: a) inoculum concentration; b) temperature (10°C and 42°C); and c) atmosphere (aerobic and microaerobic). From the data obtained, it was notorious that there were significant differences ($P < 0.001$) found for the different strains tested, inoculum concentrations, temperatures tested, and atmosphere conditions (Supplementary Tables 1 and 2). The strain was the main factor that interacted significantly with the other factors under study ($P < 0.001$).

Independently of the conditions under study, the strains *C. jejuni* 46E, *C. jejuni* 61C, *C. coli* 65B, and the reference NCTC 11168 presented a significantly different biofilm formation behavior from all the other *Campylobacter* strains (supplementary Table 1). The strain *C. jejuni* 104B presented on average the lowest optical density (**OD**) values (0.0259) when compared to all tested strains, while the strains 46E and 61C had on average the highest OD values (0.318 and 0.359, respectively, $P < 0.001$). The strains *C. coli* 65B and the control reference strain (*C. jejuni* NCTC 11168) had OD values between 0.190 and 0.198, also significantly different ($P < 0.001$) from the OD values obtained by the other strains when screened for biofilm formation ability.

The two different inoculum concentrations of *Campylobacter* tested in the biofilm ability screening assay (10^3 CFU/mL and 10^6 CFU/mL) resulted in significant differences in OD values obtained (Supplementary Table 2). In general, when the strains were inoculated at lower levels (10^3 cfu/mL), they obtained significantly higher OD values than when inoculation was performed at higher concentration levels (10^6 cfu/mL). The lower concentration seems to have facilitated the growth and posterior biofilm formation, independently of the conditions tested. Results from SAS statistical analysis indicated that regardless of the temperature and atmospheric conditions tested, high inoculum concentration led to a significant decrease in adherence levels ($P < 0.05$, Figure 2) for the *C. jejuni* strains 105-1BR, 105B, 106A, 106B, 46E, and 61C. On the other hand, it had the reverse effect for the *C. jejuni* strains 20C and NCTC 11168, and *C. coli* strain 65B. A possible explanation for the detrimental effect of high inoculum concentration on some strains could be the rapid nutrient scarcity. Culture media was not renewed. Therefore, a higher inoculum concentration likely resulted in a more aggressive competition for nutrients; this could have led the bacterial population to enter a starvation state faster, not leaving enough time for the population to make a transition to the biofilm state. In other words, maybe high competition, and a nutrient-deficient medium, inhibited adherence in these strains.

On the contrary, in the strains that obtained higher adherence levels when incubated with a higher inoculum concentration, it could be speculated that bacterial populations entered the death phase very fast, leading to the accumulation of biomass composed of dead cells. This deposit of dead cells could have facilitated the adherence of surviving cells, leading to higher ODs obtained in crystal violet assay. Overall, inoculum concentration was shown to be an essential factor affecting OD, since it appeared to highly influence adherence for some strains.

It is interesting to notice that the aerobic and microaerobic atmosphere conditions also significantly influenced ($P < 0.001$) the formation of biofilm (Supplementary Table 2). The microaerobic condition promoted the formation of biofilms independently of the strains and temperature of incubation. However, it was possible to notice that the strains 105E, 106E, 22A, 61C, 65B and 46E were tolerant to the aerobic condition, and on average, they did not have significantly different OD values when incubated under microaerobic condition. These strains had the trend to present higher OD values under aerobiosis.

The behavior of *Campylobacter* spp. strains at an incubation temperature of 10°C, under the different inoculum concentrations and atmosphere conditions tested (aerobic and microaerobic), is presented in Figures 3 A and 3B. This temperature was, in general, significantly less favorable for the formation of biofilm

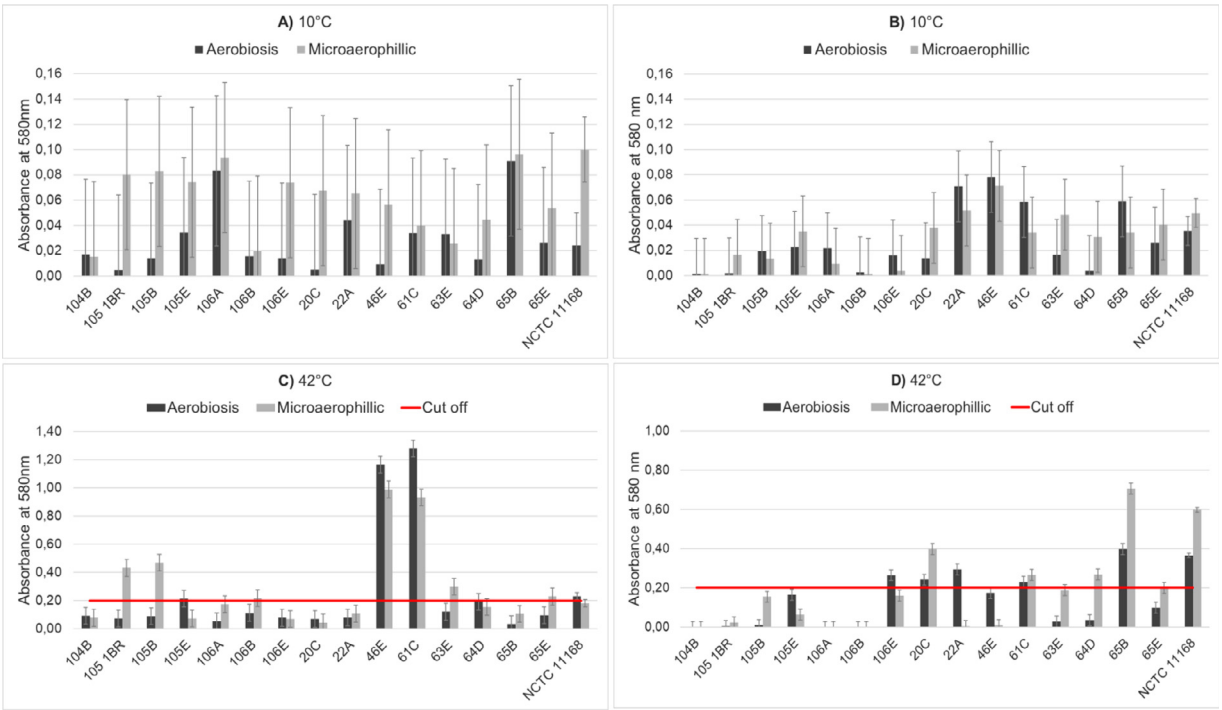

Figure 3. Effect of inoculum concentration, temperature, and atmosphere conditions on biofilm formation by 15 *Campylobacter* strains and the control *C. jejuni* NCTC 11168. (A) Assay 1, 10^3 CFU/mL inoculum concentration, at 10°C, under aerobic or microaerophilic conditions. (B) Assay 2, 10^6 CFU/mL inoculum concentration, at 10°C, under aerobic or microaerophilic conditions. (C) Assay 1, 10^3 CFU/mL inoculum concentration, at 42°C, under aerobic or microaerophilic conditions. (D) Assay 2, 10^6 CFU/mL inoculum concentration, at 42°C, under aerobic or microaerophilic conditions. Biofilm formation was assessed by CV staining. Every strain was inoculated with tree repetitions (tree consecutive lanes) on one occasion. Error bars represent the standard error of the mean. Cut off line represents the value (OD = 0.2) from which the OD values obtained were significantly different ($P < 0.0005$).

when compared with the results obtained under 42°C (Supplementary Table 2, and Figures 3C and 3D). Regarding the impact caused by the temperatures tested (10°C and 42°C), for both low and high inoculum assays, adherence levels were significantly lower at 10°C.

Other authors have studied *C. jejuni* biofilms quantified via crystal violet staining (Reeser et al., 2007; Teh et al., 2017) and reported similar values to those observed in our study at 10°C. However, the minimum OD cut-off value for strains classified according to their biofilm formation ability ranges between 0.2 and 0.35 according to Melo et al. (2017) and Zhang et al. (2017). For that reason, and from the data observed in the strains tested, it should not be assumed that biofilm formation occurred at 10°C, only some level of adherence, which could indicate an initial step for biofilm formation.

When low inoculum concentration was tested under aerobiosis at 10°C, the strain with the highest adherence level was *C. coli* 65B (OD = 0.091, Figure 3A), followed by *C. jejuni* 106A (OD = 0.083). These strains also presented the highest adherence levels under microaerobic atmosphere; however, the OD values obtained were not significantly different when compared to those obtained by all the other strains. Compared to the control strain used (*C. jejuni* NCTC 11168), these strains obtained very similar adherence levels under microaerobic atmosphere, and at least 3 times higher under aerobiosis (Figure 3A). Nevertheless, these differences were not significant given the large standard errors achieved (Figure 3A).

When a higher inoculum concentration was tested (10^6cfu/g), at 10°C (Figure 3B), the OD mean values obtained were also low in general (OD < 0.1). The strains with the highest level of adherence under aerobiosis were *C. jejuni* 46E (OD = 0.078) and *C. jejuni* 22A (OD = 0.071). Under microaerobic atmosphere, *C. jejuni* 46E and *C. jejuni* 22A were also the strains with highest level of adherence. Both these strains obtained adherence levels that surpassed the ones obtained for the control strain *C. jejuni* NCTC 11168 (OD = 0.05; Figure 3B), however the difference was also not significant (Figure 3B).

Campylobacter adherence results obtained at 42°C are presented in Figures 3C) and D). In assay 1 (low inoculum concentration) (Figure 3C), the strains that obtained the highest biofilm formation were *C. jejuni* 46E and *C. jejuni* 61C ($P < 0.0001$) for both aerobiosis and microaerobic conditions, at 42°C. The highest OD values were obtained under aerobiosis for strains 46E (OD =1.163) and 61C (OD =1.280). The capacity for biofilm formation presented by these 2 strains was remarkable. Furthermore, *C. jejuni* 46E and *C. jejuni* 61C were included in the 2 largest clusters (III and VIII) assigned in the *flaA*-RFLP dendrogram. These genotypes presented the *flaA*-RFLP pattern that was most commonly obtained in the collection of isolates (n = 143). Besides, inside these clusters, there is evidence of the persistence of these genotypes over time. Indeed, *C. jejuni* 46E genotype was associated with samples collected in January 2019 and June 2019, and *C. jejuni* 61C genotype was obtained for samples collected in November 2018 and March 2019. The ability to form biofilms found in this study, corroborates the capacity to persist in the slaughterhouse environment.

With high inoculum concentration (Figure 3D), at 42°C, the highest producer of biofilm (P value < 0.0001), under both aerobic (OD = 0.397) and microaerobic (OD = 0.706) atmospheres, was *C. coli* 65B. The oxygen has significantly inhibited biofilm formation in this strain since adherence level was significantly higher under microaerobic atmosphere (P value < 0.0001). The *C. jejuni* strains 22A, 106E, 20C, and 61C, had the highest adherence levels after *C. coli* 65B. They all presented OD mean values >0.2, either under aerobic or microaerobic atmosphere. All these strains were in clusters in which there was evidence of persistence during time. This result is very relevant because it suggests that biofilm formation capacity supported *fla*A-RFLP typing results since the strains that adhered the most (at 42°C) were also the ones with a *fla*A-RFLP genotype that persisted during the period studied (November 2018 to June 2019).

If we assume a cut-off value of 0.2 as the threshold for biofilm formation, then in the low concentration assay, 8 strains would be classified as biofilm producing bacteria. As for the high concentration assay, seven strains would also be in the same category. Besides, many strains demonstrated high tolerance to cold and aerobic atmosphere during the 72 h of incubation, such that viable cell counts were maintained with little to no decrease after 3 d of incubation at 10°C under aerobiosis, as assessed in the laboratory by classical plate counting methods with a selective medium (results not shown). These results concluded that some strains have biofilm formation ability, and several factors can facilitate their permanence at the slaughterhouse environment due to their demonstrated tolerance to low temperature and aerobic environment. Since the slaughterhouse has different sectors where temperatures can reach 30°C, such as the area of bleeding and depluming, these are the zones facilitating *Campylobacter* permanence through biofilms in equipment, regardless of *Campylobacter* adhesion capacity in subsequent zones (clean areas) with lower ambient temperature (<12°C).

When comparing ODs obtained by each strain under each atmosphere condition, significant differences were found at 42°C when a high inoculum concentration was tested. The significant differences found suggest that oxygen stimulated adherence in *C. jejuni* strains 106E, 22A, 46E, and *C. coli* 105E, but inhibited it in *C. jejuni* strains 105B, 20C, 63E, 64D, 65E, NCTC 11168 and in *C. coli* 65B (P value < 0.01). Under low inoculum concentration, at 42°C, the strains *C. jejuni* 46E and 61C had significantly higher OD levels under aerobiosis. The aerotolerance of some strains seems to have been a determinant factor for *Campylobacter* persistence and formation of biofilm. In fact, it has already been reported (Mouftah et al., 2021) that hyper aerotolerant strains of *Campylobacter* (strains that survived for more than 24 h

under aerobic shaking at 200 rpm) may present significantly higher adhesion capability and biofilm formation potential at 42°C when compared to aerotolerant and aerosensitive strains.

In general, there was high variability in adherence levels among the different strains tested. In other words, levels of adherence were strain-dependent. Similar results were found by Kim et al. (2017) when testing 78 *Campylobacter* isolates obtained from raw chicken. Most isolates (64 isolates, 82%) did not have the ability to form a biofilm (OD < 0.05), as assessed by the crystal violet staining method, and only 14 isolates presented some level of biofilm formation. This study demonstrated that wild strains from chicken vary significantly in their biofilm formation ability on polystyrene (Kim et al., 2017).

As for the association between biofilm assay results and antibiotic resistance rates, it is essential to discuss the adherence levels of the strains that had the highest resistance levels. *C. jejuni* 106E, the strain with the highest number of acquired resistances, reached an OD value higher than 0.2 in assay two at 42°C under aerobiosis, even though it was not among the strains that presented the highest adherence levels.

The strains that reached the highest adherence levels indicating biofilm formation ability were among the ones found to have resistance to the lowest number of antibiotics tested.

CONCLUSIONS

The *flaA*-typing allowed the selection of isolates with different band patterns for antibiotic susceptibility tests and biofilm assays, demonstrating the existence of genetic variability in *Campylobacter*. The clustering analysis of *flaA*-RLFP genotypes indicated that strains with the same genetic pattern were present during a long period in the slaughterhouse (from November 2018 to May 2019).

From the 15 strains tested, three strains exhibited significant biofilm formation ability, that is, *C. jejuni* 46E, *C. jejuni* 61C, and *C. coli* 65B. The ability to form biofilm was strain dependent. In both biofilm assays performed (testing low and high inoculum concentration), there was high variability in adherence levels among the different strains tested. The aerobic environment also affected the biofilm's formation ability depending on the strain considered. In some cases, oxygen seemed to have been a stimulus for biofilm formation. Low temperature (10°C) was clearly a detrimental factor for *Campylobacter* biofilm formation since adherence levels at this temperature was consistently lower than 0.1 (OD580 nm). Somewhat surprisingly, inoculum concentration was also shown to have an impact on biofilm formation in some strains. When the various factors studied are considered, it can be concluded that the conditions mimicking lower temperatures found in the clean zones of the slaughterhouse (10°C under aerobiosis), did not favor biofilm formation. On the other hand, a different behavior can be expected when temperatures in the slaughterhouse are higher, such that three strains (*C. jejuni* 46E, *C. jejuni* 61C, and *C.coli* 65B) demonstrated to have significant biofilm formation ability at 42°C. These results conclude that some wild strains from poultry origin likely have the ability to form biofilms in the first areas of the slaughterhouse (bleeding, scalding, defeathering) where temperatures can reach nearly 30°C. Consequently, if biofilms are formed, contamination sources are established, and cross contamination can occur in the next steps (evisceration, washing, chilling). Wild *Campylobacter* strains can also tolerate low temperature and aerobic environments; therefore, it remains very important to keep *Campylobacter* contamination levels low, even before the entrance of poultry in the slaughter line.

Also, if the adherence levels obtained at 10°C indicate some potential ability to attach to the surfaces present in the environment, it is essential to implement effective cleaning and disinfection procedures since biofilms are the next step after adhesion.

ACKNOWLEDGMENTS

We thank projects GO228 PDR2020-1.0.1-FEADER-PDR2020-101-031254 funded by National Funds and co-funded by the European Union, and UIDP/00276/2020 (CIISA) supported by National Funds through FCT-Foundation for Science and Technology.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101586.

REFERENCES

Abd El-Hamid, M. I., N. K. Abd El-Aziz, M. Samir, E. El-Naenaeey, Y. sayed, E. M. Abo Remela, R. A. Mosbah, and M. M Bendary. 2019. Genetic diversity of *Campylobacter jejuni* isolated from avian and human sources in Egypt. Front. Microbiol. 10:1–14.

Birk, T., H. Ingmer, M. T. Andersen, K. Jørgensen, and L. Brøndsted. 2004. Chicken juice, a food-based model system suitable to study survival of *Campylobacter jejuni*. Lett. Appl. Microbiol. 38:66–71.

Brown, H. L., M. Reuter, L. J. Salt, K. L. Cross, R. P. Betts, and A. H. M. van Vliet. 2014. Chicken juice enhances surface attachment and biofilm formation of *Campylobacter jejuni*. Appl. Environ. Microbiol. 80:7053–7060.

Carpentier, B., and O. Cerf. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. J. Appl. Microbiol. 75:499–511.

CA-SFM [Comite de L'antibiogramme de la Société Française de Microbiologie]. (2020). Comité de l'antibiogramme de la Société Française de microbiologie recommandations 2020 V.1.1 Avril. https://www.sfm-microbiologie.org/wp-content/uploads/2020/04/CASFM2020_Avril2020_V1.1.pdf Accessed Nov. 2020.

CLSI [Clinical and Laboratory Standards Institute]. 2018. . 4th ed CLSI supplement VET08: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals: *38*CLSI, Wayne, PA.

Corry, J., Jørgensen, F., Purnell, G., James, C., Pinho, R., & James, S. J. (2017). Reducing Campylobacter cross-contamination during poultry processing. Pages 1−159 in research project technical report FS990010 (M01039). https://www.food.gov.uk/research/research-projects/reducing-campylobacter-cross-contamination-during-poultry-processing Accessed Nov. 2020.

Damjanova, I., M. Jakab, T. Farkas, J. Mészáros, Z. Galántai, I. Turcsányi, A. Bistyák, Á. Juhász, J. Pászti, I. Kiss, and G. Kardos. 2011. From farm to fork follow-up of thermotolerant campylobacters throughout the broiler production chain and in human cases in a Hungarian county during a ten-months period. Int. J. Food Microbiol. 150:95–102.

Dhakal, J., C. S. Sharma, R. Nannapaneni, C. D. McDaniel, T. Kim, and A. Kiess. 2019. Effect of chlorine-induced sublethal oxidative stress on the biofilm-forming ability of salmonella at different temperatures, nutrient conditions, and substrates. J. Food Prot. 82:78–92.

Duarte, A., A. Santos, J. Benoliel, F. Domingues, and M. Oleastro. 2013. A infeção humana por Campylobacter em Portugal: alguns dados epidemiológicos. Observ. Bolet. Epidemiol. (INSA) 7:17–19. .http://hdl.handle.net/10400.18/1679 Accessed Nov. 2020.

Efimochkina, N. R., I. B. Bykova, Y. M. Markova, Y. V. Korotkevich, V. V. Stetsenko, L. P. Minaeva, and S. A. Sheveleva. 2017. Formation of biofilms by foodborne pathogens and development of laboratory in vitro model for the study of Campylobacter genus bacteria based on these biofilms. Bull. Exp. Biol. Med. 162:474–478.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). 2020. The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018. EFSA J 18:6007.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). 2021. The European Union One Health 2019 Zoonoses Report. EFSA J. 19:6406.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards). 2013. Scientific opinion on carbapenem resistance in food animal ecosystems. EFSA J. 11:3501.

EN/ISO 10272-1. 2017. Microbiology of the Food Chain — Horizontal Method for Detection and Enumeration of Campylobacter spp. — Part 1: Detection Method. International Organization for Standardization, Genève, Switzerland.

EN/ISO 10272-2. 2017. Microbiology of the Food Chain — Horizontal Method for Detection and Enumeration of Campylobacter spp. — Part 2: Colony-Count Technique. International Organization for Standardization, Genève, Switzerland.

EUCAST (The European Committee on Antimicrobial Susceptibility Testing). (2020). Breakpoint tables for interpretation of MICs and zone diameters version 10.0. https://www.eucast.org/clinical_breakpoints/ Accessed Nov. 2020.

European Union Comission. 2017. Commission Regulation (EU) 2017/1495 of 23 August 2017 amending Regulation (EC) No 2073/2005 as regards Campylobacter in broiler carcases. Off. J Eur Union, L 218:1–6.

Farfán, M., N. Lártiga, M. B. Benavides, R. Alegría-Morán, L. Sáenz, C. Salcedo, and L. Lapierre. 2019. Capacity to adhere to and invade human epithelial cells, as related to the presence of virulence genes in, motility of, and biofilm formation of Campylobacter jejuni strains isolated from chicken and cattle. Can. J. Microbiol. 65:126–134.

Fraqueza, M. J., A. Martins, A. C. Borges, M. H. Fernandes, M. J. Fernandes, Y. Vaz, R. J. B. Bessa, and A. S. Barreto. 2014. Antimicrobial resistance among Campylobacter spp. strains isolated from different poultry production systems at slaughterhouse level. Poult. Sci. 93:1578–1586.

Fraqueza, M. J., S. A. Ribeiro, S. C. Pereira, M. H. Fernandes, M. J. Fernandes, and A. S. Barreto. 2016. Genetic and antibiotic resistance profiles of thermophilic Campylobacter spp. isolated from quails (Coturnix coturnix japonica) in a Portuguese slaughterhouse. Food Control 59:337–344.

García-Sánchez, L., B. Melero, I. Jaime, M. Rossi, I. Ortega, and J. Rovira. 2019. Biofilm formation, virulence and antimicrobial resistance of different Campylobacter jejuni isolates from a poultry slaughterhouse. Food Microbiol 83:193–199.

Hagiya, H., K. Kimura, I. Nishi, H. Yoshida, N. Yamamoto, Y. Akeda, and K. Tomono. 2018. Emergence of carbapenem non-susceptible Campylobacter coli after long-term treatment against recurrent bacteremia in a patient with X-linked agammaglobulinemia. Intern. Med. J. 57:2077–2080.

Kayman, T., S. Abay, F. Aydin, and O. Şahin. 2019. Antibiotic resistance of Campylobacter jejuni isolates recovered from humans with diarrhoea in Turkey. J. Med. Microbiol. 68:136–142.

Kim, J. C., E. Oh, J. Kim, and B. Jeon. 2015. Regulation of oxidative stress resistance in Campylobacter jejuni, a microaerophilic foodborne pathogen. Front. Microbiol. 6:1–12.

Kim, S. H., C. Park, E. J. Lee, W. S. Bang, Y. J. Kim, and J. S. Kim. 2017. Biofilm formation of Campylobacter strains isolated from raw chickens and its reduction with DNase I treatment. Food Control 71:94–100.

Lamas, A., P. Regal, B. Vázquez, J. Miranda, A. Cepeda, and C. Franco. 2018. Salmonella and Campylobacter biofilm formation: a comparative assessment from farm to fork. J. Sci. Food Agric. 98:4014–4032.

Lienhard, A., and S. Schäffer. 2019. Extracting the invisible: obtaining high quality DNA is a challenging task in small arthropods. Peer J. 7 Article e6753.

Melo, R. T., E. P. Mendonça, G. P. Monteiro, M. C. Siqueira, C. B. Pereira, P. A. B. M. Peres, H. Fernandez, and D. A. Rossi. 2017. Intrinsic and extrinsic aspects on Campylobacter jejuni Biofilms. Front. Microbiol. 8:1–15.

Mouftah, S. F., J. F. Cobo-Díaz, A. Álvarez-Ordóñez, A. Mousa, J. K. Calland, B. Pascoe, S. K. Sheppard, and M. Elhadidy. 2021. Stress resistance associated with multi-host transmission and enhanced biofilm formation at 42°C among hyper-aerotolerant generalist Campylobacter jejuni. Food Microbiol 95:1–11.

Oh, E., K. J. Andrews, L. M. McMullen, and B. Jeon. 2019. Tolerance to stress conditions associated with food safety in Campylobacter jejuni strains isolated from retail raw chicken. Sci. Rep. 9:1–9.

O'Toole, G. A. 2011. Microtiter dish biofilm formation assay. J. Vis. Exp. 47:10–11.

Pang, X., and H. G. Yuk. 2018. Effect of Pseudomonas aeruginosa on the sanitizer sensitivity of Salmonella enteritidis biofilm cells in chicken juice. Food Control 86:59–65.

Perez-Arnedo, I., and E. Gonzalez-Fandos. 2019. Prevalence of Campylobacter spp. in poultry in three Spanish farms, a slaughterhouse and a further processing plant. Foods 8:111.

Reeser, R. J., R. T. Medler, S. J. Billington, B. H. Jost, and L. A. Joens. 2007. Characterization of Campylobacter jejuni biofilms under defined growth conditions. Appl. Environ. Microbiol. 73:1908–1913.

Rossi, D. A., R. T. Melo, E. P. Mendonça, and G. P. Monteiro. 2017. Chapter 5: Biofilms of Salmonella and Campylobacter in the poultry industry. Pages 93−106 in Poultry Science. M. Manafi, ed. IntechOpen.https://wwwintechopen.com/books/poultry-science/biofilms-of-salmonella-and-campylobacter-in-the-poultry-industry Accessed Nov. 2020.

Samosornsuk, W., M. Asakura, E. Yoshida, T. Taguchi, K. Nishimura, B. Eampokalap, V. Phongsisay, W. Chaicumpa, and S. Yamasaki. 2007. Evaluation of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the identification of Campylobacter strains isolated from poultry in Thailand. Microbiol. Immunol. 51:909–917.

Sanders, S. Q., D. H. Boothe, J. F. Frank, and J. W. Arnold. 2007. Culture and detection of Campylobacter jejuni within mixed microbial populations of biofilms on stainless steel. J. Food Prot. 70:1379–1385.

Skarp, C. P. A., M. L. Hänninen, and H. I. K. Rautelin. 2016. Campylobacteriosis: the role of poultry meat. Clin. Microbiol. Infect. 22:103–109.

Solomon, E. B., and D. G. Hoover. 1999. Campylobacter jejuni: a bacterial paradox. J. Food Saf. 19:121–136.

Teh, A. H. T., S. M. Lee, and G. A. Dykes. 2014. Does Campylobacter jejuni form biofilms in food-related environments? Appl. Environ. Microbiol. 80:5154–5160.

Teh, A. H. T., S. M. Lee, and G. A. Dykes. 2016. The influence of prior modes of growth, temperature, medium, and substrate surface on biofilm formation by antibiotic-resistant Campylobacter jejuni. Curr. Microbiol. 73:859–866.

Teh, A. H. T., S. M. Lee, and G. A. Dykes. 2017. Identification of potential Campylobacter jejuni genes involved in biofilm formation by EZ-Tn5 Transposome mutagenesis. BMC Res. Notes 10:1–7.

Vidal, A. B., F. M. Colles, J. D. Rodgers, N. D. McCarthy, R. H. Davies, M. C. J. Maiden, and F. A. Clifton-Hadley. 2016. Genetic diversity of Campylobacter jejuni and Campylobacter coli isolates from conventional broiler flocks and the impacts of sampling strategy and laboratory method. Appl. Environ. Microbiol. 82:2347–2355.

Wagle, B. R., A. Upadhyay, I. Upadhyaya, S. Shrestha, K. Arsi, R. Liyanage, K. Venkitanarayanan, D. J. Donoghue, and A. M Donoghue. 2019. Trans-Cinnamaldehyde, eugenol and carvacrol reduce Campylobacter jejuni biofilms and modulate expression of select genes and proteins. Front. Microbiol. 10:1837.

Wassenaar, T. M., and D. G. Newell. 2000. Genotyping of Campylobacter spp. Appl. Environ. Microbiol. 66:1–9.

Whitehouse, C. A., S. Zhao, and H. Tate. 2018. Antimicrobial resistance in Campylobacter species: mechanisms and genomic epidemiology. Adv. Appl. Microbiol. 103:1–47.

Wieczorek, K., E. Denis, and J. Osek. 2015. Comparative analysis of antimicrobial resistance and genetic diversity of Campylobacter from broilers slaughtered in Poland. Int. J. Food Microbiol. 210:24–32.

Zbrun, M. V., A. Romero-Scharpen, C. Olivero, J. A. Zimmermann, E. Rossler, L. P. Soto, D. M. Astesana, J. E. Blajman, A. Berisvil, L. S. Frizzo, and M. L. Signorini. 2017. Genetic diversity of thermotolerant Campylobacter spp. isolates from different stages of the poultry meat supply chain in Argentina. Rev. Argent. Microbiol. 49:235–241.

Zhang, T., J. Dong, Y. Cheng, Q. Lu, Q. Luo, G. Wen, G. Liu, and H. Shao. 2017. Genotypic diversity, antimicrobial resistance, and biofilm-forming abilities of Campylobacter isolated from chicken in Central China. Gut Pathog 9:1–10.

Zhong, X., Q. Wu, J. Zhang, Z. Ma, J. Wang, X. Nie, Y. Ding, L. Xue, M. Chen, S. Wu, X. Wei, and Y. Zhang. 2020. Campylobacter jejuni biofilm formation under aerobic conditions and inhibition by ZnO nanoparticles. Front. Microbiol. 11:207.