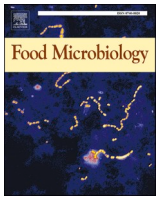
Food Microbiology 117 (2024) 104397

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Phase variation modulates the multi-phenotypes displayed by clinical *Campylobacter jejuni* strains

Irene Ortega-Sanz, Carolina Bocigas, Beatriz Melero, Jordi Rovira *

Department of Biotechnology and Food Science, University of Burgos, Burgos, Spain

ARTICLE INFO

Keywords:
Campylobacteriosis
Aerotolerance
Oxidative stress
Motility
Biofilm formation
Genomics

ABSTRACT

The high incidence and prevalence of *Campylobacter* spp. in the food supply chain entail the importance to understand their mechanisms developed to withstand harsh environmental conditions encountered. Different stress conditions and phenotypic approaches were evaluated to study the behaviour of five clinical *C. jejuni* isolates with different genotypes, including the tolerance to oxygen and the oxidants hydrogen peroxide and cumene hydroperoxide, the motility and the ability to form biofilm on polystyrene and stainless steel at different temperatures and atmospheres. Whole Genome Sequencing was performed to analyse the occurrence of 216 genes involved in these mechanisms plus phase variation. The isolates showed high tolerance to oxygen and peroxide stress with different swimming motility performances and biofilm formation abilities. Aerotolerance was related with a reduced sensitive to peroxide stress and a loss of motility that promotes biofilm formation depending on the material surface. Comparative genomics did not reveal any clear gene pattern, although phase variation occurring during host infection was observed to be crucial for the modulation of the different survival mechanisms adopted by the bacteria. These findings reveal that the bacteria can combine diverse and complex strategies in an efficient manner to survive and persist in the environment.

1. Introduction

Campylobacter spp. is the leading cause of foodborne bacterial gastroenteritis around the world with over 127,000 human cases annually in the EU in 2021 (EFSA and ECDC, 2022). Noteworthy, the COVID-19 pandemic and the withdrawal of the UK from the EU caused a drop in the number of reported campylobacteriosis cases in 2021 with respect to the annual average of 2017–2019 of 36.1%. The infective dose needed to cause disease is often low, typically fewer than 500 organisms, and higher dose may be required for acute illness (Kothary and Babu, 2001). Typical symptoms include fever, abdominal pain, nausea, vomiting and diarrhoea (which is frequently bloody). Although a great number of campylobacteriosis remain without symptoms, the level of immunity of the host is important in the development of severe complications, such as chronic inflammatory diseases (Louwen and Hays, 2013).

The major natural reservoir of *Campylobacter* spp. is poultry, from where the bacteria can spread rapidly via feces through a flock of birds in a broiler house (Rovira et al., 2006). The transmission of *Campylobacter* is also caused by consumption of contaminated food or water, undercooked poultry or raw milk and cross-contamination events in domestic kitchens (Cardoso et al., 2021). During transmission through the environment and the food supply chain, the bacteria must survive stressful conditions, particularly high oxygen levels, temperature shifts, freeze-thaw, heat shocks, osmotic stress and antimicrobial or disinfectant agents (Oh et al., 2018). Specific phenotypes, such as the capability to withstand oxidative stress and form biofilm, are among the major strategies developed by the bacteria to survive under these conditions (Karki et al., 2018). In fact, bacteria encased in biofilms are reported to be 1000-fold more resistant to disinfectants and antimicrobials than their planktonic (free-swimming) counterparts, which has become a serious problem for the food industry (Fux et al., 2005). To overcome the unfavourable conditions, the bacteria have developed molecular mechanisms, such as enzymes for defence against oxidative stress like the superoxide dismutase (SOD), catalase (KatA), cytochrome *c* peroxidases (Cj0358 and Cj0020c) and alkyl hydroperoxide reductase (AhpC) (Atack and Kelly, 2009). In contrast, biofilm formation is a complex process that occurs via multiple steps involving several gene functions, such as surface proteins, flagella and quorum sensing (Reeser et al., 2007). Furthermore, altered gene expression and phenotypic variation

* Corresponding author.
E-mail address: jrovira@ubu.es (J. Rovira).

https://doi.org/10.1016/j.fm.2023.104397
Received 14 February 2023; Received in revised form 15 September 2023; Accepted 3 October 2023
Available online 9 October 2023

has been observed within the biofilm formation (Tram et al., 2020b). However, it is still an enigma how the bacteria adopt their molecular mechanisms to resist under these hostile conditions.

Previous studies have focused mostly on the study of *Campylobacter* behaviour under specific stress conditions. The aim of this study is to investigate the genomic characteristics related to various survival strategies of five clinical *Campylobacter jejuni* isolates of different lineages through different phenotypic approaches, including aerotolerance, resistance to oxidative stresses, motility and biofilm formation. Better understanding of the survival strategies would help combat *Campylobacter* contamination.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In this study, five clinical *Campylobacter jejuni* strains were selected out of 33 isolates obtained from the University Hospital of Burgos (Spain) as representative isolates of four different PFGE (Pulsed-Field Gel Electrophoresis) profiles, that were previously identified at different locations of the poultry food supply chain. Detailed information regarding these isolates is presented in Table 1.

Stock cultures were stored at −80 °C in 20% glycerol. *C. jejuni* strains were grown on blood agar (BA) plates containing Nutrient Broth No. 2 (Oxoid) with 1.5% bacteriological agar (Oxoid) supplemented with 5% (v v^{-1}) defibrinated sheep blood (Oxoid) under microaerobic conditions (at 41.5 °C for 48 h) prior to use in assays.

2.2. Aerotolerance assay

Aerotolerance assay was carried out according to Oh et al. (2015a) with slight modifications. Briefly, *C. jejuni* strains were subcultured in Mueller-Hinton (MH) broth (Oxoid) and incubated at 37 °C overnight in a microaerobic atmosphere. Bacterial suspensions were prepared to a final concentration of ~10^7 CFU mL^{-1} and incubated at 37 °C with shaking at 200 rpm under aerobic conditions. Samples were taken after 0 h, 12 h and 24 h for serial dilution and CFU counting on BA. Experiments were performed in triplicate and repeated three times.

2.3. Oxidative stress assay

Susceptibility to oxidative stress was performed as described by Oh et al. (2015b) with slight modifications. Briefly, single bacterial cells were subcultured on BA at 37 °C for 24 h under microaerobic conditions and harvested into 2 mL MH broth. Bacterial suspensions were diluted to an OD_{600} of 1 (approximately 10^8 CFU mL^{-1}). Next, 0.5 mL of each strain were exposed to 1 mL of cumene hydroperoxide (CHP) (Sigma-Aldrich, Burlington, MA, USA) at a final concentration of 0.05% (v v^{-1}) (equivalent to 3.35 mM) and to 1 mL of hydrogen peroxide (H_2O_2) (VWR, Radnor, PA, USA) at a final concentration of 5 mM, all for 1 h at 37 °C under microaerobic conditions with shaking at 200 rpm. Samples were taken after 0 min, 15 min, 30 min and 60 min for serial dilution and CFU counting on BA. Experiments were performed in triplicate and repeated three times.

2.4. Motility agar assay

Motility of *C. jejuni* strains was assessed for aerobic and microaerobic cultures using a motility agar assay (Upadhyay et al., 2017) with slight modifications. Briefly, *C. jejuni* strains were subcultured in MH broth and incubated at 37 °C overnight in a microaerobic atmosphere. The inocula were prepared to a final concentration of ~10^7 CFU mL^{-1} and incubated aerobically and microaerobically at 37 °C during 24 h. Then, 5 μL of this suspension were stabbed into a 0.4% (w v^{-1}) MH agar plate, without touching the bottom. Suspensions were allowed to dry on the agar surface and incubated at 37 °C for 48 h. Motility proficiency was determined by measuring the diameter of the swim halo around the point of inoculation. Strains were classified as nonmotile (no growth zone), motile (up to 4 cm of diameter) or hypermotile (more than 4 cm of diameter). Experiments were performed in triplicate and repeated three times.

2.5. Quantitative biofilm formation assays

The ability of *C. jejuni* strains to form biofilm was determined on two different surfaces polystyrene 6-well cell culture plates (9,6 cm^2 $well^{-1}$) (Nunclon Delta Surface) (Thermo Scientific, Massachusetts, USA) and stainless steel coupons (rectangle 2 × 1 cm, type 1.4301 according to European Standard EN 10088-1 with a type 2B finish according to European Standard EN 10088-2), at three different temperatures 25 °C, 30 °C and 37 °C, under aerobic and microaerobic conditions. The stainless steel coupons were dry sterilized by treatment at 175 °C for 2 h and stored in sterilized aluminium foil until use. The crystal violet (CV) staining method used was an adaption based on Stepanović et al. (2007) with modifications. Briefly, single bacterial cells were subcultured on BA at 41.5 °C for 48 h under microaerobic conditions. After incubation, cells were harvested into 2 mL MH broth and the stationary-phase culture was diluted to an OD_{590} of 0.7 (approximately 7.5 $\log_{10}$ CFU mL^{-1}). A sterile stainless steel coupon was placed at the bottom of each well for the stainless steel biofilm formation assay and the diluted bacteria were then inoculated into the microtiter plates (3 mL per well). For the negative control, wells were filled only with MH broth. After incubation of plates statically for 96 h under the tested conditions, bacterial growth media and cells were removed, and wells were washed with 3 mL of sterile distilled water without disturbing the adherent biofilm. The remaining attached bacteria forming the biofilm layer were heat-fixed during 30 min at 60–70 °C following by staining with 1 mL of 0.1% crystal violet for 30 min at room temperature. Excess stain was removed, and wells were rinsed two times with 1 mL of distilled water. Once the microplates were air-dried at room temperature, stainless steel coupons were transferred to clear 6-well microtiter plates (Nunclon Delta Surface, Thermo Scientific), and the dye bound to the adherent bacterial cells in all wells was resolubilized using 1 mL of 80% alcohol 20% acetone $^{-1}$ solution per well for at least 30 min without shaking. The contents of each well were thoroughly mixed and 100 μL of the homogeneous suspension were transferred to a 96-well microtiter plate (Nunclon Delta Surface) (Thermo Scientific) for OD measurement at 590 nm in an Epoch spectrophotometer (BioTek, Winooski, USA) by using the Gen5 2.00 software. The results were interpreted based on the formula of

Table 1
Information data of strains selected.

Strain	Origin	Sex	Age	Hospitalization	ST/CC[a]	PFGE poultry supply chain[b]
H249	Clinical isolate	Male	1	No	904/607	Slaughterhouse
H518	Clinical isolate	Male	5	No	354/354	Farm
H529	Clinical isolate	Male	78	Yes	677/677	Retail
H660	Clinical isolate	Female	13	No	148/21	Hospital
H661	Clinical isolate	Male	11	Yes (perimyocarditis)	148/21	Hospital

[a] ST/CC, Sequence Type/Clonal Complex.
[b] PFGE (Pulsed-Field Gel Electrophoresis), for each isolate, the location in the poultry supply chain where the same PFGE profile was found is indicated.

Stepanović et al. (2007). The cut-off value for the negative control (ODc) was calculated for each material surface by using the mean OD_{590} of all negative control wells plus three standard deviations of all negative control wells. The strains were then categorized as non- (OD ≤ ODc), weak (ODc < OD ≤ 2 x ODc), moderate (2 x ODc < OD ≤ 4 x ODc) or strong (OD > 4 x ODc) biofilm formers. Experiments were performed in duplicate and repeated three times.

2.6. Genomic DNA extraction, Whole Genome Sequencing and genome annotation

Genomic DNA extraction was performed using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions and purity was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were constructed with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced using MiSeq platform (Illumina) generating 2 × 300 bp paired-end reads. The in-house pipeline CamPype (Ortega-Sanz et al., 2023a) was followed to filter and assemble the reads. Briefly, reads were filtered using Trimmomatic v0.39 (Bolger et al., 2014) and extended using FLASH v1.2.11 (Magoč and Salzberg, 2011), followed by *de novo* assembly through SPAdes v3.14.0 (Bankevich et al., 2012). Draft genomes were reordered against the reference genome *C. jejuni* NCTC 11168 using progressiveMAUVE v2.4.0 (Darling et al., 2010) and annotated using Prokka v1.14.6 (Seemann, 2014) with the same reference genome as previously mentioned.

2.7. Core genome MLST (cgMLST)

Phylogenetic comparison of the five *C. jejuni* genome sequences was performed by constructing a Neighbor-Joining (NJ) tree based on the distance matrix of the core genome of all strains and the reference genome *C. jejuni* NCTC 11168 (GCA_000009085.1). The core genome MLST (cgMLST) analysis was performed using chewBBACA v2.8.5 (Silva et al., 2018) following the pubMLST *Campylobacter jejuni/coli* cgMLST scheme of 1343 loci (Cody et al., 2017) (last updated on September 12, 2022) with the default Prodigal training file provided for genome *C. jejuni* NCTC 11168-BN148 (https://www.ncbi.nlm.nih.gov/nuccore/HE978252). Allele profile data was subsequently used to obtain the cgMLST allele distance matrix by cgmlst-dists v0.4.0 (https://github.com/tseemann/cgmlst-dists). MEGA X v10.2.5 (Tamura et al., 2021) was used to calculate the NJ tree from the distance matrix using the reference genome *C. jejuni* NCTC 11168 as root.

2.8. Comparative genomics analysis

An in-house database of 216 genes of *C. jejuni* NCTC 11168 involved in aerotolerance, oxidative stress, motility and biofilm formation (Supplementary Table S1) was blasted twice against the draft and annotated genomes through BLAST 2.10.1+ (Altschul et al., 1990) to identify genetic differences among strains. Results were manually corrected for overlapping hits based on analyzing Bidirectional Best Hits (BBHs) of genes using the *orthologr* R package (Drost et al., 2015) and gene synteny for highly similar genes. Truncations were considered as gene presence if the hit occurred at contigs boundaries. The strain *C. jejuni* NCTC 11168 was used as control in all BLAST analyses. Adittionaly, phase variation was analysed for the hypervariable genes included in the database (Supplementary Table S1) (Parkhill et al., 2000).

2.9. Statistical analyses

Statistical analysis was carried out Statgraphics Centurion XIX. One-way analysis of variance (ANOVA) was used to compare growth means for aerotolerance assay data, oxidative stress assay data and motility agar assay data. The ability of *C. jejuni* isolates to form biofilm on each material (polystyrene and stainless steel) under two variables atmosphere with two levels (aerobic and microaerobic) and temperature with three levels (25 °C, 30 °C and 37 °C) was determined by multifactor analysis of variance (multifactor ANOVA). The model also considered interactions between strains, temperature and atmosphere. Fisher's LSD (Least Significant Difference) test was applied to establish differences between means at 95% significance level.

3. Date availabilitys

The sequenced genomes were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession numbers GCA_947044865 (H249), GCA_947044855 (H518) and GCA_947044845 (H529). Genome assembly for strains H660 and H661 can be found at GCA_947044875 and GCA_947044885, respectively.

4. Results

4.1. Response to aerotolerance

The aerotolerance of the *C. jejuni* isolates was studied by growing them aerobically with vigorous shaking at 200 rpm. The isolates were clustered into two groups depending on the levels of aerotolerance: (i) the aerotolerant group (strains H249 and H661, 40%) that maintained viability 12~24 h of aerobic shaking with a final mean concentration of 5.64 ± 0.83 $\log_{10}$ CFU mL^{-1}, and (ii) the hyper-aerotolerant group (strains H518, H529 and H660, 60%) in which isolates remained viable even after 24 h of aerobic shaking with a final mean concentration of 1.94 ± 0.61 $\log_{10}$ CFU mL^{-1} (Fig. 1).

4.2. Response of C. jejuni strains to oxidative stress

The *C. jejuni* isolates were tested for sensitivity to CHP (an organic hydroperoxide) and H_2O_2 (an inorganic hydroperoxide) at four time points (0 min, 15 min, 30 min and 60 min). All isolates behaved similar to both oxidants with a significant loss of bacterial viability over time, which was remarkably higher during the first 15 min (Fig. 2). An immediate toxic effect to all strains caused by CHP and H_2O_2 was observed, except for strain H661 to both oxidants and strain H529 to CHP. While the initial sensitivity to H_2O_2 was higher compared to CHP exposure, sensitivity to CHP was higher at the next time points (15 min, 30 min and 60 min). At 60 min, CHP reduced bacterial viability in >6.25 ± 0.32 $\log_{10}$ CFU mL^{-1}, whereas 5.89 ± 0.60 $\log_{10}$ CFU mL^{-1} were reduced by

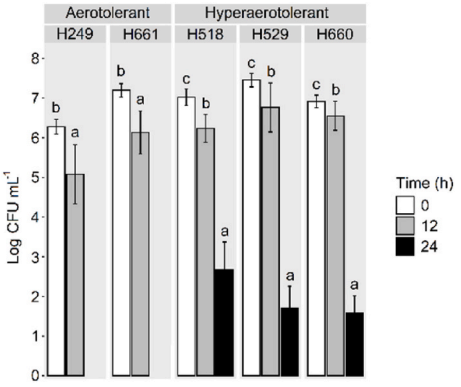


Fig. 1. Aerotolerance levels of the *Campylobacter jejuni* isolates tested in this study. The aerotolerant isolates (left) maintained viability 12~24 h of aerobic shaking, while hyper-aerotolerant isolates (right) remained viable after 24 h of aerobic shaking. Each bar indicates the mean, and the error bars represent the standard deviation of the data for three independent experiments. The aerotolerance tests were repeated three times in each experiment. Statistical significance for each isolate was analysed with one-way ANOVA ($p < 0.05$). Detection limit of experiment was 1 $\log_{10}$ CFU/mL.

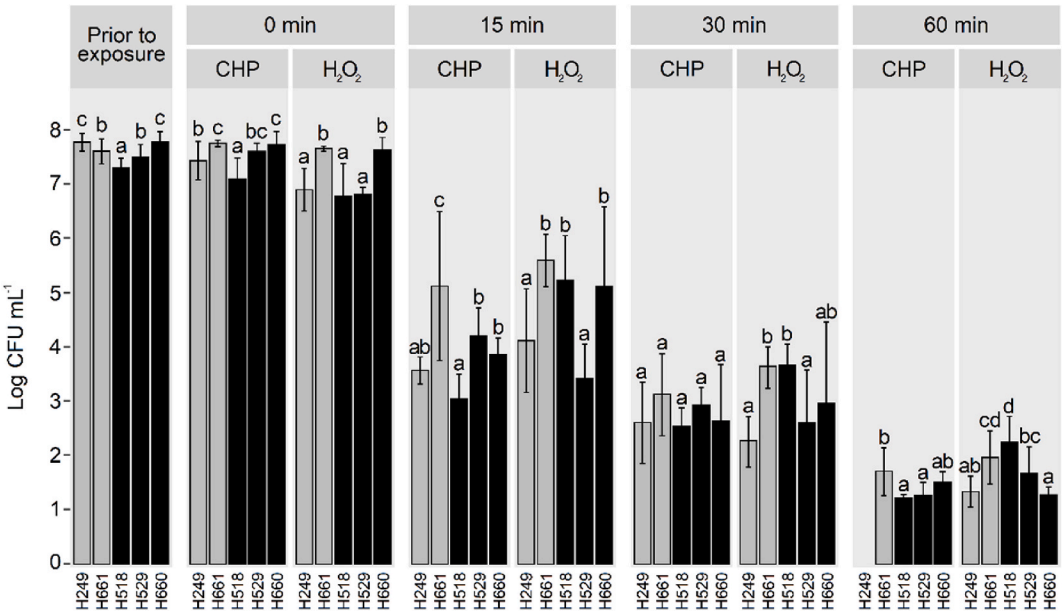


Fig. 2. Effect of oxidative stress on the survival of the *Campylobacter jejuni* isolates tested in this study. *C. jejuni* strains were incubated with 0.05% CHP (cumene hydroperoxide) or 5 mM H_2O_2 (hydrogen peroxide) for 1 h at 37 °C and bacterial survival assessed at various time points. Each bar indicates the mean, and the error bars represent the standard deviation of the data for three independent experiments. The oxidative stress tests were repeated three times in each experiment. Statistical significance at each time point for each oxidant was analysed with one-way ANOVA ($p < 0.05$). Detection limit of experiment was 1 $\log_{10}$ CFU/mL. Grey, aerotolerant; black, hyper-aerotolerant.

H_2O_2. Therefore, all strains were more resistant to H_2O_2 at 60 min, except strain H660, and only the strain H249 was completely removed down to the detection limit (1 $\log_{10}$ CFU mL^{-1}) after exposure to CHP during 60 min. The tested *C. jejuni* isolates showed decreasing resistance to oxidative stress after 60 min caused by CHP in the following order H661 > H660 > H529 > H518 > H249, and by H_2O_2 in the following order H518 > H661 > H529 > H249 > H660.

4.3. Motility test

Analysis of the motility of the *C. jejuni* strains in MH agar at 37 °C showed differences between aerobic and microaerobic atmospheres. Although the swimming motility of all isolates was completely abolished under aerobiosis after 24 h, and even at 12 h, significant differences were observed between all isolates under microaerobiosis (Table 2). The strain H660 was the only nonmotile isolate and the remaining ones showed decreasing motility in the following order H249 > H661 > H518 > H529, with strain H249 being hypermotile (>4 cm).

4.4. Biofilm formation under different conditions

The effect of various types of materials (polystyrene and stainless steel), temperatures (25 °C, 30 °C and 37 °C) and atmospheres (aerobiosis and microaerobiosis) in biofilm formation by the *C. jejuni* isolates of study was evaluated using the CV staining method. Levels of biofilm formation on the surfaces of materials were significantly different among strains and temperatures ($p < 0.001$), and also between atmospheres on polystyrene ($p < 0.001$) (Supplementary Table S2). Furthermore, the effects of temperature on both materials were strain- and atmosphere-dependent ($p < 0.05$), with increasing biofilm formation in aerobiosis at upper temperatures, except at 25 °C on polystyrene and at 37 °C on stainless steel, when increased biofilm formation occurred in microaerobiosis. Tables 3 and 4 show the OD_{590} values obtained for each strain in the biofilm assays on polystyrene and stainless steel, respectively. The OD_{590} cutoff value (ODc) established to consider biofilm formation by the strains tested for polystyrene assays was 0.317. Therefore, all the strains were able to produce biofilm in polystyrene assays. Of those, the strain H660 showed the highest biofilm formation, with no significant difference in the OD with strain H518, whereas the strain H249 was the least biofilm producer. Moreover, biofilm formation was noted to be higher at 37 °C, followed by 30 °C and then 25 °C, and in aerobiosis. It was especially interesting in the case of strain H660 at 37 °C in aerobiosis, which showed an OD_{590} value almost two times higher than the other strains (Table 3). In stainless steel assays, the strains that showed OD_{590} values lower than 0.209 were considered as non-biofilm formers, that included a total of two strains not producing biofilm at 25 °C in both atmospheres and at 30 °C in microaerobiosis (Table 4). The strains H660 and H661 showed the highest biofilm formation on stainless steel, whereas the strain H529 was the least biofilm producer. Besides, biofilm formation was noted to be higher at 37 °C, while there was no significant difference in the OD between 25 °C and 30 °C.

Table 2
Swarming motility on semisoft agar of the *Campylobacter jejuni* isolates tested in this study under microaerobic atmosphere.

Type	Strain	Growth zone (cm)
Hypermotile	H249	4.3 ± 0.4^{e}
Motile	H661	3.2 ± 0.3^{d}
	H518	2.6 ± 0.3^{c}
	H529	1.4 ± 0.3^{b}
Nonmotile	H660	0^{a}

Results are shown as mean ± standard deviation. Different letters indicate significant differences ($p < 0.05$).

4.5. Genomic characterization of C. jejuni strains

The five *C. jejuni* genomes were assembled *de novo*. Overall genome characteristics are shown in Table 5. To assess the genomic relatedness among the isolates, pairwise ANI values were computed, with $ANI_b > 97$ for all genome pairs.

Phylogenetic tree created from the cgMLST schema formed three different branches (Fig. 3). The strains H660 and H661 belonging to the same ST-148 and CC21 were grouped together and showed close

Table 3
Biofilm formation on polystyrene plates at the three different incubation temperatures (25 °C, 30 °C and 37 °C) used on the two different atmospheres (aerobiosis and microaerobiosis) by the *Campylobacter jejuni* isolates tested in this study.

Motility		25 °C		30 °C		37 °C	
	Strain	A	M	A	M	A	M
Hipermotile	H249	$_{A}^{a}$ 0.337 ± 0.032 (+)	$_{A}^{a}$ 0.356 ± 0.020 (+)	$_{A}^{b}$ 0.630 ± 0.052 (+)	$_{A}^{a,*}$ 0.373 ± 0.022 (+)	$_{A}^{b}$ 0.650 ± 0.045 (++)	$_{A}^{b,*}$ 0.517 ± 0.047 (+)
Motile	H661	$_{D}^{a}$ 0.464 ± 0.050 (+)	$_{A}^{a}$ 0.387 ± 0.055 (+)	$_{A}^{ab}$ 0.600 ± 0.063 (+)	$_{AB}^{a,*}$ 0.423 ± 0.084 (+)	$_{A}^{b}$ 0.689 ± 0.188 (++)	$_{C}^{b}$ 0.733 ± 0.065 (++)
	H518	$_{B}^{a}$ 0.395 ± 0.040 (+)	$_{B}^{a,*}$ 0.467 ± 0.061 (+)	$_{B}^{c}$ 0.963 ± 0.065 (++)	$_{B}^{a*}$ 0.471 ± 0.073 (+)	$_{A}^{b}$ 0.644 ± 0.056 (++)	$_{B}^{b}$ 0.623 ± 0.043 (+)
	H529	$_{BC}^{a}$ 0.402 ± 0.026 (+)	$_{A}^{a}$ 0.375 ± 0.037 (+)	$_{A}^{b}$ 0.666 ± 0.063 (++)	$_{C}^{b}$ 0.572 ± 0.078 (+)	$_{A}^{b}$ 0.744 ± 0.202 (++)	$_{A}^{b}$ 0.555 ± 0.053 (+)
Nonmotile	H660	$_{CD}^{a}$ 0.451 ± 0.034 (+)	$_{C}^{b,*}$ 0.607 ± 0.058 (+)	$_{A}^{a}$ 0.563 ± 0.121 (+)	$_{A}^{a,*}$ 0.355 ± 0.024 (+)	$_{B}^{b}$ 1.113 ± 0.074 (++)	$_{C}^{c,*}$ 0.738 ± 0.030 (++)

Results are expressed as the means ± standard deviation of the data. Different letters (lower-case) in the same row and the same atmosphere (A, aerobiosis; M, microaerobiosis) indicate significant differences ($p < 0.05$). Asterisks indicate significant differences ($p < 0.05$) between the 2 atm for the same temperature. Different letters (upper-case) in the same column indicate significant differences ($p < 0.05$). Comparisons of the OD values produced by bacterial biofilms to the ODc value were used to classify the strains. (−), non-biofilm former (OD ≤ ODc); (+), weak biofilm former (ODc < OD ≤ 2 x ODc); (++), moderate biofilm former (2 x ODc < OD ≤ 4 x ODc). The cut-off OD (ODc) value was defined as three standard deviations above the mean OD of the negative control.

Table 4
Biofilm formation on stainless steel coupons at the three different incubation temperatures (25 °C, 30 °C and 37 °C) used on the two different atmospheres (aerobiosis and microaerobiosis) by the *Campylobacter jejuni* isolates tested in this study.

Motility		25 °C		30 °C		37 °C	
	Strain	A	M	A	M	A	M
Hipermotile	H249	$_{B}^{a}$ 0.229 ± 0.021 (+)	$_{AB}^{a,*}$ 0.191 ± 0.019 (−)	$_{AB}^{a}$ 0.253 ± 0.013 (+)	$_{BC}^{a,*}$ 0.215 ± 0.024 (+)	$_{A}^{a}$ 0.269 ± 0.043 (+)	$_{B}^{b}$ 0.318 ± 0.039 (+)
Motile	H661	$_{C}^{a}$ 0.285 ± 0.031 (+)	$_{C}^{a,*}$ 0.228 ± 0.026 (+)	$_{BC}^{a}$ 0.272 ± 0.024 (+)	$_{AB}^{a,*}$ 0.203 ± 0.014 (−)	$_{B}^{b}$ 0.351 ± 0.034 (+)	$_{D}^{b,*}$ 0.464 ± 0.035 (++)
	H518	$_{A}^{a}$ 0.194 ± 0.008 (−)	$_{BC}^{a,*}$ 0.216 ± 0.013 (+)	$_{A}^{a}$ 0.220 ± 0.009 (+)	$_{CD}^{ab}$ 0.242 ± 0.028 (+)	$_{A}^{b}$ 0.283 ± 0.031 (+)	$_{AB}^{b}$ 0.276 ± 0.030 (+)
	H529	$_{A}^{a}$ 0.196 ± 0.019 (−)	$_{A}^{a}$ 0.184 ± 0.016 (−)	$_{A}^{a}$ 0.218 ± 0.019 (+)	$_{A}^{a,*}$ 0.181 ± 0.016 (−)	$_{A}^{b}$ 0.279 ± 0.036 (+)	$_{A}^{b}$ 0.255 ± 0.012 (+)
Nonmotile	H660	$_{C}^{a}$ 0.281 ± 0.034 (+)	$_{C}^{a}$ 0.234 ± 0.027 (+)	$_{C}^{a}$ 0.299 ± 0.042 (+)	$_{D}^{a,*}$ 0.247 ± 0.021 (+)	$_{A}^{a}$ 0.283 ± 0.016 (+)	$_{C}^{b,*}$ 0.367 ± 0.032 (+)

Results are expressed as the means ± standard deviation of the data. Different letters (lower-case) in the same row and the same atmosphere (A, aerobiosis; M, microaerobiosis) indicate significant differences ($p < 0.05$). Asterisks indicate significant differences ($p < 0.05$) between the 2 atm for the same temperature. Different letters (upper-case) in the same column indicate significant differences ($p < 0.05$). Comparisons of the OD values produced by bacterial biofilms to the ODc value were used to classify the strains. (−), non-biofilm former (OD ≤ ODc); (+), weak biofilm former (ODc < OD ≤ 2 x ODc); (++), moderate biofilm former (2 x ODc < OD ≤ 4 x ODc). The cut-off OD (ODc) value was defined as three standard deviations above the mean OD of the negative control.

Table 5
Summary of the most common features of the *Campylobacter jejuni* isolates tested in this study.

		H249	H518	H529	H660	H661
MLST	Sequence type (ST)	ST-904	ST-354	ST-677	ST-148	ST-148
	Clonal complex (CC)	CC607	CC354	CC677	CC21	CC21
Assembly	Completeness (%)	99.0	99.7	99.0	99.7	99.7
	Contigs	26	26	31	24	25
	Genome length (bp)	1,759,864	1,765,374	1,640,863	1,677,040	1,666,126
	Genome coverage (X)	317	325	312	800	763
	DNA G + C content (%)	30.16	30.30	30.34	30.39	30.39
Annotation	CDS	1829	1873	1700	1711	1708
	Hypothetical proteins	403	461	314	297	294
	rRNA	5	6	5	6	6
	tRNA	40	40	40	40	40
	CRISPR sequences	1	1	0	0	0

MLST, multilocus sequence typing. CDS, coding DNA sequence.

relationship (two cgMLST loci differences) and the same distance to the reference *C. jejuni* NCTC 11168, while strains H529, H518 and H249 showed higher genetic diversity, as previously shown by the 3 distinct STs and CCs identified (Table 5), but specially strain H529, that formed a separate branch.

A total of 216 genes directly or indirectly related to aerotolerance/oxidative stress, motility or/and biofilm formation were searched for their presence/absence in the five *C. jejuni* isolates and compared to the *C. jejuni* NCTC 11168 reference genome. From these, 182 genes were present in all genomes and differences in the gene occurrence among the five *C. jejuni* isolates of study were found for 33 of them, while the phase-variable (PV) gene *cj1318* (*maf1*) (unknown motility accessory factor) located in the flagellin glycosylation locus was only present in *C. jejuni* NCTC 11168 (Supplementary Table S1) (Fig. 3). Moreover, four PV genes present in all genomes (*flgR*, *cj1295*, *cj1310c* and *maf7*) showed different phase sate among isolates, that summed to the 10 PV genes

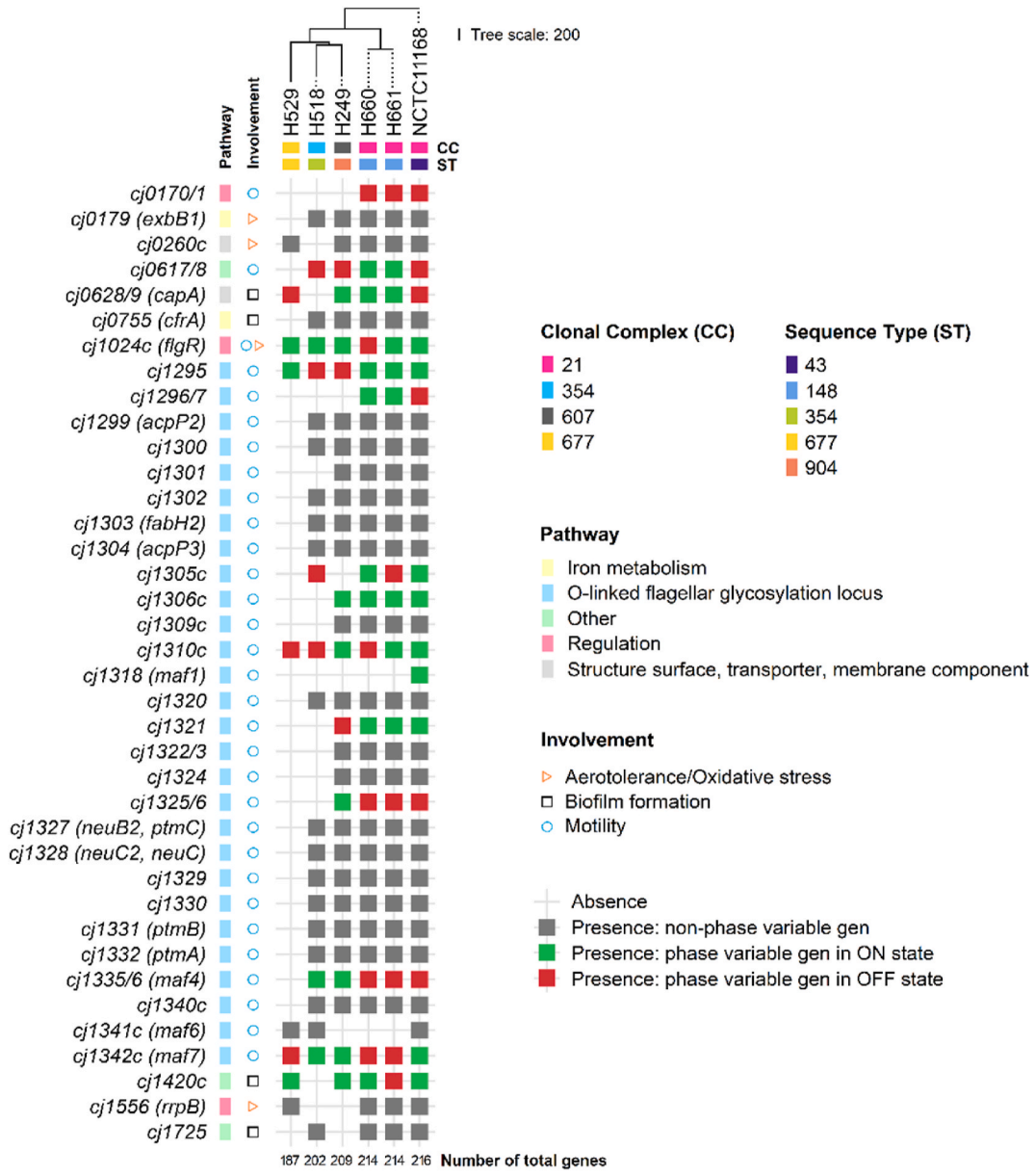


Fig. 3. Differences detected between the 216 genes analysed involved in biofilm formation, motility and oxidative stress/aerotolerance among the *Campylobacter jejuni* isolates tested in this study. The NJ cgMLST tree is shown at the top. ST and CC are shown in colored boxes for each strain. Pathways are shown in colored boxes for each gen and symbols indicate the phenotype test in which the genes are involved. The genes showing no differences among all genomes are not shown. Different colored squares represent the presence of genes and color code distinguish between non-phase-variable genes (grey) and phase-variable genes (green and red). ST, Sequence Type. CC, Clonal Complex.

showing different prevalence pattern (*cj0170/1*, *cj0617/8*, *capA*, *cj1296/7*, *cj1305c*, *cj1306c*, *cj1321*, *cj1325/6*, *maf4* and *cj1420c*) constitute the remaining 14 PV genes of study. That was, 37.8% (14 out 37) of the genes with differences among the five *C. jejuni* isolates were phase-variable, and 12 of them affected motility (*cj0170/1*, *cj0617/8*, *flgR*, *cj1295*, *cj1296/7*, *cj1305c*, *cj1306c*, *cj1310c*, *cj1321*, *cj1325/6*, *maf4* and *maf7*). Between 187 and 214 genes could be identified among the five *C. jejuni* isolates with decreasing gene frequency in the following order H660 = H661 > H249 > H518 > H529.

5. Discussion

Despite the common perception of the sensibility of *C. jejuni* to oxygen, results demonstrated that aerotolerant and hyperaerotolerant strains of *C. jejuni* are prevalent in cases of human infection, specially the hyperaerotolerant strains. In agreement with this finding, various studies reported the isolation of aerotolerant and hyperaerotolerant *C. jejuni* strains isolated from clinical source with larger proportions of hyperaerotolerant strains compared to aerotolerant strains (58.6–85.7%) (Oh et al., 2018; Mouftah et al., 2021). However, for *C. jejuni* isolated from poultry, higher sensitivity rates to oxygen have been reported with a proportion of 93.4% isolates not surviving at 12 h (Karki et al., 2018). This suggests that clinical *C. jejuni* isolates would be capable of overcoming high oxygen concentration. Type VI secretion system (T6SS) was suggested to confer tolerance to oxygen when in combination with other features (O'Kane and Connerton, 2017), but, the strain H249, that harboured the complete T6SS, did not demonstrate higher tolerance to oxygen compared to the other *C. jejuni* strains, which none of them possessed this secretion system.

Aerotolerance is known to be associated with increased oxidative

stress resistance (Oh et al., 2015a, 2018). This study demonstrated that aerotolerant and hyperaerotolerant clinical *C. jejuni* strains were highly resistant to peroxide stress, even with oxidants concentrations higher than those tested by Oh et al. (2015b) in *C. jejuni* strains isolated from raw chicken meats (more than 30 times the concentration of CHP and 5 times the concentration of H_2O_2). Therefore, the passage of *C. jejuni* through human host might induce factors which render the bacteria less sensitive to the reactive oxygen species (ROS) produced by the human immune response during infection (Atack and Kelly, 2009), and therefore would be of public health importance as *C. jejuni* would be more virulent and pathogenic than bacteria isolated from other sources. In fact, a population genetics analysis of 121 strains of *C. jejuni* causing human campylobacteriosis revealed that clinical *C. jejuni* isolates were primarily clustered in multistress-tolerant clades (Oh et al., 2018). The changes that might explain the different aerotolerance and peroxide stress phenotypes could be associated with genome rearrangement of genes involved in iron metabolism (*exbB1*), regulation (*flgR* and *rrpB*) and membrane structure (*cj0260c*). This suggests that a complex genomic system involving different pathways and phase variable genes (*flgR*) affect the behaviour of *C. jejuni* under oxygen and peroxide exposure.

Motility is an important virulence trait of *C. jejuni* for colonization of the intestinal tract (Guerry, 2007). This study showed that aerotolerance was associated with a loss of motility under oxygen-enriched environment, in contrast to a study that showed no relation between aerotolerance and motility for *C. coli* strain OR12 (O'Kane and Connerton, 2017). However, the majority of *C. jejuni* strains were motile in microaerobiosis, indicating that the bacteria might perform better inside the host cell where motility may equate to increased virulence. The different swimming performances observed for all the *C. jejuni* strains of study in microaerobiosis indicates that this phenotype is variable, even for closely related strains of the same lineage as observed for H660 and H661 (CC21). However, when the nonmotile H660 strain was spotted onto semi-solid agar after 24 h and 48 h incubation in liquid medium, short non-uniform spreading sectors up to 0.5 cm were observed sometimes from the point of inoculation, that means that the motile phenotype of strain H660 spread on average only a 13% compared to the swarming motility of its clonal isolate H661. Interestingly, this pattern was observed in a non-spreading mutant of *C. jejuni* NCTC 11168 after 96 h agar incubation and such behaviour was the result of a single nucleotide deletion in an 8T tract within *cj1179c* (*fliR*), that codes for a structural protein of the flagellar Type III secretion system (T3SS) (Sher et al., 2020). However, both strains H660 and H661 shared the complete Open Reading Frame (ORF) of *fliR*. Differences between the nonmotile H660 strain and the motile H661 strain were observed for other phase variables genes coding for a regulation protein (FlgR), hypothetical proteins involved in the O-linked flagellin glycosylation (Cj1305c and Cj1310c) and a putative methyltransferase (Cj1420c). And all these genes except *cj1420c* were involved in motility. These findings highlight that motility can be occasionally revertible over time to some extent and that this phenotypic alteration could be enabled by phase variation of genes involved in motility. However, compared to the other motile strains, only the OFF state of the *flgR* response regulator, which is essential for activation of σ^{54}-dependent flagellar genes and therefore flagella biosynthesis, seemed to affect negatively to motility in *C. jejuni*. Ewing et al. (2009) observed that the filament was not assembled in a *flgR* mutant, suggesting that the phase variation of *flgR* affect the completion of flagella resulting in a time-revertible *C. jejuni* motility. However, the highly reduced swimming performance of strain H660, when motile, indicates that motility is under complex regulation in *C. jejuni*. Nonetheless, the combination of ON/OFF states between strains H660 and H661 modulated virulence through host colonization (Ortega-Sanz et al., 2023b), that ultimately lead to different phenotypes in *C. jejuni*, suggesting that genomic variants rather than gene rearrangements might be responsible for the different survival mechanisms exhibited by the bacteria.

Bacterial motility also indirectly contributes to *C. jejuni* resistance to oxidative stress as disruption of flagellar rotation causes an imbalance proton gradient through electron leakage along the electron transport chain that increases the generation of ROS within the cell, that ultimately increases the sensitivity to oxidative stress (Flint et al., 2014). However, in this study, the strain H249, that was the most sensitive to oxidative stress, was hypermotile and, on the contrary, the nonmotile strain H660 was resistant to superoxide stress.

The ability to form biofilm was determined on two commonly materials in the food industry, such as polystyrene and stainless steel. The *C. jejuni* isolates formed biofilm to various degrees on both materials with a relative increase in biofilm formation on polystyrene as previously observed Wagle et al. (2019). Properties of materials such as roughness, wettability, or hydrophobicity influence the ability of cells to attach or detach to a surface (Van Houdt and Michiels, 2010). Thus, the hydrophobic property of polystyrene compared to the moderately hydrophilic property with a negative surface charge of stainless steel favoured stronger interactions between the hydrophobic cell surface of bacteria, such as flagella and exopolysaccharides, and polystyrene surface (Donlan, 2002; Ban et al., 2014). Therefore, cleaning and disinfection procedures in the food industry of not only polystyrene surfaces, such as water suppliers, feeding stations or containers for poultry transportation, but also stainless steel equipment, need to be effective knowing the versatility of *C. jejuni* to develop biofilm on such materials. Incubation temperature and oxygen concentration also influenced biofilm formation as higher temperatures and aerophilic growth enhanced biofilm formation, which is in agreement with previous publications (Oh et al., 2016; García-Sánchez et al., 2019; Araújo et al., 2022), except at 37 °C on stainless steel. These results indicate that several environmental factors, including oxygen atmosphere and temperature, influence the ability of *C. jejuni* to form biofilms, and that optimal *C. jejuni* growth conditions facilitate permanence of the bacteria through biofilms depending on material surface. Moreover, biofilm formation by *C. jejuni* differed between strains, which is consistent with earlier data (García-Sánchez et al., 2019; Araújo et al., 2022). However, there was no correlation in the strains showing high or low biofilm formation on different material surfaces, as reported Lajhar et al. (2018), except for strain H660. Therefore, biofilm production on one surface might not correlate with biofilm formation on a different surface, despite the scientific principle is the same for both methods. Nonetheless, this could be the result of the differences observed for the genes *cfrA* (ferric enterobactin receptor of the cell membrane involved in iron metabolism), *cj1725* (putative periplasmic protein), and the hypervariable genes *capA* (autotransporter associated with adhesion and invasion of epithelial cells) and *cj1420c* (putative methyltransferase). Therefore, the outer parts of the cell seem to be important for biofilm formation. Moreover, the protein Cj1725, that was absent in strain H249 and H529, might be related with weaker biofilm formation capabilities.

Biofilm formation capability is also linked to aerotolerance and oxidative stress (Oh et al., 2016). The increased biofilm formation ability to oxygen exposure is consequence of the accumulation of ROS, that stimulates the development of this mechanism of resistance. The results of this study confirmed a significant increased biofilm formation potential ($p < 0.05$) on polystyrene for the hyperaerotolerant isolates (H518, H529 and H660) compared to the aerotolerant isolates (H249 and H661) that was enhanced under aerobic conditions, as previously reported Reuter et al. (2010) and Mouftah et al. (2021). However, on stainless steel, the aerotolerant isolates were the ones that showed a significant increased biofilm formation potential and, on average, the aerotolerant and hyperaerotolerant isolates did not have significantly different OD values when incubated under different atmospheres. Hence, the ability of *C. jejuni* to form biofilm favoured by oxygen tolerance depends on the material surface.

Moreover, the flagella play a crucial role during biofilm formation (Reeser et al., 2007). However, the loss of motility observed for all the motile *C. jejuni* strains under oxygen-enriched environment indicates

that flagella are not essential for biofilm formation but could participate in the initial attachment of *C. jejuni* to surfaces as suggested Reuter et al. (2010). Actually, it was demonstrated an inverse relationship between motility and biofilm formation as reduced motility of *C. jejuni* promotes higher auto-agglutination of bacteria, that is a precursor of biofilm formation (Rahman et al., 2014; Tram et al., 2020a). This is consistent with the reduced and increased biofilm formation abilities on polystyrene of the hypermotile H249 strain and the nonmotile H660 strain, respectively. However, on stainless steel, biofilm formation was independent of motility, suggesting that this process is subject to a complex genetic regulation. Thus, the strain H529, which was defective in flagellin glycosylation due to a reduced size of the O-linked flagellar glycosylation locus (*cj1293-cj1342c*) comprising only 23 genes, demonstrate a reduced biofilm formation potential on stainless steel whilst its motility was unaffected, as observed Howard et al. (2009) on glass. This proves that biofilm formation on stainless steel might be subject to flagellin glycosylation. Moreover, Goon et al. (2003) revealed that *C. coli* defective in flagella glycosylation were more fragile than filaments fully glycosylated, which could explain the reduced motility of strain H529.

6. Conclusions

The multi-phenotypes observed reveal that *C. jejuni* is able to combine different strategies in an efficient manner to survive and persist along the different environments existing in the food supply chain and that these survival mechanisms are directly or indirectly associated. The identification of gene patterns probably linked with the phenotypic traits observed was challenging due to the different phenotypes observed among the *C. jejuni* strains. However, various point mutations in phase variable genes between closely related isolates with different phenotypes were identified suggesting that phase variation is more likely to modulate the phenotype of *C. jejuni* under hostile conditions. This suggests that the survival mechanisms adopted by the bacteria are diverse and complexly regulated. Further research would be required, such as the identification of SNPs in core genes, analysis of differential gene expression or the execution of genome-wide association studies (GWAS) with larger numbers of isolates to facilitate comparative statistical studies, that would provide more information into adaptive mechanism of *C. jejuni* in harsh environments.

Funding statement

This work was supported by “La Caixa” Foundation and Caja Burgos Foundation, under agreement LCF/PR/PR18/51130007. Ortega-Sanz I received a predoctoral grant from the Junta of Castile and León, cofinanced by the Ministry of Education of the Government of Castile and León and the European Social Fund.

CRediT authorship contribution statement

Irene Ortega-Sanz: Conceptualization, Methodology, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Beatriz Melero:** Conceptualization, Funding acquisition, Project administration, Supervision, Resources, Writing – review & editing. **Jordi Rovira:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

We declare that there are not conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104397.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Araújo, P.M., Araújo, P.M., Batista, E., Fernandes, M.H., Fernandes, M.J., Gama, L.T., Fraqueza, M.J., 2022. Assessment of biofilm formation by Campylobacter spp. isolates mimicking poultry slaughterhouse conditions. Poultry Sci. 101 (2), 101586 https://doi.org/10.1016/j.psj.2021.101586.

Atack, J.M., Kelly, D.J., 2009. Oxidative stress in Campylobacter jejuni: responses, resistance and regulation. Future Microbiol. 4 (6), 677–690. https://doi.org/10.2217/fmb.09.44.

Ban, G.H., Yoon, H., Kang, D.H., 2014. A comparison of saturated steam and superheated steam for inactivation of Escherichia coli O157:H7, Salmonella Typhimurium, and Listeria monocytogenes biofilms on polyvinyl chloride and stainless steel. Food Control 40, 344–350. https://doi.org/10.1016/j.foodcont.2013.12.017.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V. M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol.: J. Computat. Molecular Cell Biol. 19 (5), 455–477. https://doi.org/10.1089/cmb.2012.0021.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Cardoso, M.J., Ferreira, V., Truninger, M., Maia, R., Teixeira, P., 2021. Cross-contamination events of Campylobacter spp. in domestic kitchens associated with consumer handling practices of raw poultry. Int. J. Food Microbiol. 338, 108984 https://doi.org/10.1016/j.ijfoodmicro.2020.108984.

Cody, A.J., Bray, J.E., Jolley, K.A., McCarthy, N.D., Maiden, M., 2017. Core genome multilocus sequence typing scheme for stable, comparative analyses of Campylobacter jejuni and C. Coli human disease isolates. J. Clin. Microbiol. 55 (7), 2086–2097. https://doi.org/10.1128/JCM.00080-17.

Darling, A.E., Mau, B., Perna, N.T., 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5 (6), e11147. https://doi.org/10.1371/journal.pone.0011147.

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8 (9), 881–890. https://doi.org/10.3201/eid0809.020063.

Drost, H.G., Gabel, A., Grosse, I., Quint, M., 2015. Evidence for active maintenance of phylotranscriptomic hourglass patterns in animal and plant embryogenesis. Mol. Biol. Evol. 32 (5), 1221–1231. https://doi.org/10.1093/molbev/msv012.

EFSA and ECDC, 2022. The European union one health 2021 zoonoses report. EFSA J. 20 (12), 7666. https://doi.org/10.2903/j.efsa.2022.7666, 2022.

Ewing, C.P., Andreishcheva, E., Guerry, P., 2009. Functional characterization of flagellin glycosylation in Campylobacter jejuni 81-176. J. Bacteriol. 191 (22), 7086–7093. https://doi.org/10.1128/JB.00378-09.

Flint, A., Sun, Y.Q., Butcher, J., Stahl, M., Huang, H., Stintzi, A., 2014. Phenotypic screening of a targeted mutant library reveals Campylobacter jejuni defenses against oxidative stress. Infect. Immun. 82 (6), 2266–2275. https://doi.org/10.1128/IAI.01528-13.

Fux, C.A., Costerton, J.W., Stewart, P.S., Stoodley, P., 2005. Survival strategies of infectious biofilms. Trends Microbiol. 13 (1), 34–40. https://doi.org/10.1016/j.tim.2004.11.010.

García-Sánchez, L., Melero, B., Jaime, I., Rossi, M., Ortega, I., Rovira, J., 2019. Biofilm formation, virulence and antimicrobial resistance of different Campylobacter jejuni isolates from a poultry slaughterhouse. Food Microbiol. 83, 193–199. https://doi.org/10.1016/j.fm.2019.05.016.

Goon, S., Kelly, J.F., Logan, S.M., Ewing, C.P., Guerry, P., 2003. Pseudaminic acid, the major modification on Campylobacter flagellin, is synthesized via the Cj1293 gene. Mol. Microbiol. 50 (2), 659–671. https://doi.org/10.1046/j.1365-2958.2003.03725.x.

Guerry, P., 2007. Campylobacter flagella: not just for motility. Trends Microbiol. 15 (10), 456–461. https://doi.org/10.1016/j.tim.2007.09.006.

Howard, S.L., Jagannathan, A., Soo, E.C., Hui, J.P., Aubry, A.J., Ahmed, I., Karlyshev, A., Kelly, J.F., Jones, M.A., Stevens, M.P., Logan, S.M., Wren, B.W., 2009. Campylobacter jejuni glycosylation island important in cell charge, legionaminic acid biosynthesis, and colonization of chickens. Infect. Immun. 77 (6), 2544–2556. https://doi.org/10.1128/IAI.01425-08.

Karki, A.B., Marasini, D., Oakey, C.K., Mar, K., Fakhr, M.K., 2018. Campylobacter coli from retail liver and meat products is more aerotolerant than Campylobacter jejuni. Front. Microbiol. 9, 2951. https://doi.org/10.3389/fmicb.2018.02951.

Kothary, M.H., Babu, U.S., 2001. Infective dose of foodborne pathogens in volunteers: a review. J. Food Saf. 21 (1), 49–68. https://doi.org/10.1111/j.1745-4565.2001.tb00307.x.

Lajhar, S.A., Brownlie, J., Barlow, R., 2018. Characterization of biofilm-forming capacity and resistance to sanitizers of a range of E. coli O26 pathotypes from clinical cases and cattle in Australia. BMC Microbiol. 18 (1), 41. https://doi.org/10.1186/s12866-018-1182-z.

Louwen, R., Hays, J.P., 2013. Is there an unrecognised role for Campylobacter infections in (chronic) inflammatory diseases? World J. Clin. Infect. Dis. 3 (4), 58–69. https://doi.org/10.5495/wjcid.v3.i4.58.

Magoč, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27 (21), 2957–2963. https://doi.org/10.1093/bioinformatics/btr507.

Mouftah, S.F., Cobo-Díaz, J.F., Álvarez-Ordóñez, A., Mousa, A., Calland, J.K., Pascoe, B., Sheppard, S.K., Elhadidy, M., 2021. Stress resistance associated with multi-host transmission and enhanced biofilm formation at 42 °C among hyper-aerotolerant generalist Campylobacter jejuni. Food Microbiol. 95, 103706 https://doi.org/10.1016/j.fm.2020.103706.

O'Kane, P.M., Connerton, I.F., 2017. Characterisation of aerotolerant forms of a robust chicken colonizing Campylobacter coli. Front. Microbiol. 8, 513. https://doi.org/10.3389/fmicb.2017.00513.

Oh, E., McMullen, L., Jeon, B., 2015a. Impact of oxidative stress defense on bacterial survival and morphological change in Campylobacter jejuni under aerobic conditions. Front. Microbiol. 6, 295. https://doi.org/10.3389/fmicb.2015.00295.

Oh, E., McMullen, L., Jeon, B., 2015b. High prevalence of hyper-aerotolerant Campylobacter jejuni in retail poultry with potential implication in human infection. Front. Microbiol. 6, 1263. https://doi.org/10.3389/fmicb.2015.01263.

Oh, E., Kim, J.C., Jeon, B., 2016. Stimulation of biofilm formation by oxidative stress in Campylobacter jejuni under aerobic conditions. Virulence 7 (7), 846–851. https://doi.org/10.1080/21505594.2016.1197471.

Oh, E., Chui, L., Bae, J., Li, V., Ma, A., Mutschall, S.K., Taboada, E.N., McMullen, L.M., Jeon, B., 2018. Frequent implication of multistress-tolerant Campylobacter jejuni in human infections. Emerg. Infect. Dis. 24 (6), 1037–1044. https://doi.org/10.3201/eid2406.171587.

Ortega-Sanz, I., Barbero-Aparicio, J.A., Canepa-Oneto, A., Rovira, J., Melero, B., 2023a. CamPype: an open-source workflow for automated bacterial whole-genome sequencing analysis focused on Campylobacter. BMC Bioinf. 24 (1), 291. https://doi.org/10.1186/s12859-023-05414-w.

Ortega-Sanz, I., García, M., Bocigas, C., Megías, G., Melero, B., Rovira, J., 2023b. Genomic characterization of Campylobacter jejuni associated with perimyocarditis: a family case report. Foodborne Pathoge. Disease 20 (8), 368–373. https://doi.org/10.1089/fpd.2023.0010.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M.A., Rutherford, K. M., van Vliet, A.H., Whitehead, S., et al., 2000. The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. Nature 403 (6770), 665–668. https://doi.org/10.1038/35001088.

Rahman, H., King, R.M., Shewell, L.K., Semchenko, E.A., Hartley-Tassell, L.E., Wilson, J. C., Day, C.J., Korolik, V., 2014. Characterisation of a multi-ligand binding chemoreceptor CcmL (Tlp3) of Campylobacter jejuni. PLoS Pathog. 10 (1), e1003822 https://doi.org/10.1371/journal.ppat.1003822.

Reeser, R.J., Medler, R.T., Billington, S.J., Jost, B.H., Joens, L.A., 2007. Characterization of Campylobacter jejuni biofilms under defined growth conditions. Appl. Environ. Microbiol. 73 (6), 1908–1913. https://doi.org/10.1128/AEM.00740-06.

Reuter, M., Mallett, A., Pearson, B.M., van Vliet, A.H., 2010. Biofilm formation by Campylobacter jejuni is increased under aerobic conditions. Appl. Environ. Microbiol. 76 (7), 2122–2128. https://doi.org/10.1128/AEM.01878-09.

Rovira, J., Cencic, A., Santos, E.M., Jakobsen, M., 2006. Biological harzards. In: Luning, P.A., Devlieghere, F., Verhé, Wageningen, R. (Eds.), Safety in the Agrifood Chain. Academic Publishers, Wageningen, The Netherlands, pp. 67–143.

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30 (14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

Sher, A.A., Jerome, J.P., Bell, J.A., Yu, J., Kim, H.Y., Barrick, J.E., Mansfield, L.S., 2020. Experimental evolution of Campylobacter jejuni leads to loss of motility, rpoN (σ54) deletion and genome reduction. Front. Microbiol. 11, 579989 https://doi.org/10.3389/fmicb.2020.579989.

Silva, M., Machado, M.P., Silva, D.N., Rossi, M., Moran-Gilad, J., Santos, S., Ramirez, M., Carriço, J.A., 2018. chewBBACA: a complete suite for gene-by-gene schema creation and strain identification. Microb. Genom. 4 (3), e000166 https://doi.org/10.1099/mgen.0.000166.

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., Ruzicka, F., 2007. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica 115 (8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38 (7), 3022–3027. https://doi.org/10.1093/molbev/msab120.

Tram, G., Day, C.J., Korolik, V., 2020a. Bridging the gap: a role for Campylobacter jejuni biofilms. Microorganisms 8 (3), 452. https://doi.org/10.3390/microorganisms8030452.

Tram, G., Klare, W.P., Cain, J.A., Mourad, B., Cordwell, S.J., Day, C.J., Korolik, V., 2020b. Assigning a role for chemosensory signal transduction in Campylobacter jejuni biofilms using a combined omics approach. Sci. Rep. 10 (1), 6829. https://doi.org/10.1038/s41598-020-63569-5.

Upadhyay, A., Arsi, K., Wagle, B.R., Upadhyaya, I., Shrestha, S., Donoghue, A.M., Donoghue, D.J., 2017. Trans-cinnamaldehyde, carvacrol, and eugenol reduce Campylobacter jejuni colonization factors and expression of virulence genes in vitro. Front. Microbiol. 8, 713. https://doi.org/10.3389/fmicb.2017.00713.

Van Houdt, R., Michiels, C.W., 2010. Biofilm formation and the food industry, a focus on the bacterial outer surface. J. Appl. Microbiol. 109 (4), 1117–1131. https://doi.org/10.1111/j.1365-2672.2010.04756.x.

Wagle, B.R., Upadhyay, A., Upadhyaya, I., Shrestha, S., Arsi, K., Liyanage, R., Venkitanarayanan, K., Donoghue, D.J., Donoghue, A.M., 2019. Trans-cinnamaldehyde, eugenol and carvacrol reduce Campylobacter jejuni biofilms and modulate expression of select genes and proteins. Front. Microbiol. 10, 1837. https://doi.org/10.3389/fmicb.2019.01837.