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Beyond the plate: Genomic characterization of an extensive school *Clostridium perfringens* type F outbreak

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ABSTRACT

Objectives: *Clostridium perfringens* is one of the leading causes of food-borne outbreaks (FBO). This study explores the epidemiological and genomic features of a major FBO occurred in a Spanish school in 2023.
Methods: Through genomic analysis, the virulence factors and relatedness of *C. perfringens* strains involved in a widespread gastroenteritis outbreak were identified
Results: The FBO had a unimodal epidemic curve, affecting 526 people. The likely source was the meat in Bolognese sauce. The clinical strains, which belonged to two different clonal lineages (ST-804/cgST-804 and ST5/cgST-801), carried a wide virulence profile with multiple toxins including CPA, enterotoxin CPE, cytolytic toxin CPB2, perfringolysin, collagenase A, clostripain, hyaluronidases (NagHIJKL) and sialidases (NanIHJ). FBO strains of *C. perfringens* type F carry the *cpe* gene on the chromosome rather than on a plasmid. However, during this outbreak, the *cpe* gene, encoding the CPE enterotoxin, was mobilized by a pCPF4969-like plasmid. Susceptibility to metronidazole and vancomycin was retained. Distinct phage repertoires were observed in both lineages.
Conclusions: This is the first genomic study of a *C. perfringens* FBO in Spain, suggesting a multiple contamination sources in the same outbreak. The findings underscore substantial public health concerns related to food safety practices within large-scale user facilities.

Background

The multiple arsenal of toxins and virulence genes of *Clostridium perfringens*, together with its ability to form spores, cause severe diseases such as gas gangrene (clostridial myonecrosis), food-poisoning, enteric diseases (including antibiotics-associated diarrhea, hemorrhagic enteritis and necrotic enteritis) in various hosts [1]. *C. perfringens* produces at least 20 extracellular toxins and hydrolytic enzymes, each with different biological action mechanisms and cellular targets [2]. They are classified into seven toxinotypes (A–G), based on the production of major lethal toxins: alpha (CPA, encoded by *the cpa/plc* gene), beta (CPB, *cpb* gene), epsilon (ETX, *etx* gene), and iota (ITX, *iap* and *ibp* binary genes), along with enterotoxin (CPE, *cpe* gene) and necrotic enteritis B-like toxin (NETB, *netB* gene). Each toxinotype is associated with a specific disease in a particular host [3]. Currently, strains carrying genes encoding CPA and CPE toxins, but not CPB, ETX, ITX and NETB toxins, are classified as *C. perfringens* Type F food poisoning strains (instead type A, as before) [3]. Type F strains are primarily responsible for human food poisoning and other enteric diseases, such as antibiotics-associated diarrhea, due to the characteristic fluid accumulation and mucosal damage caused by enterotoxin CPE (*4*). Other clinically relevant accessory toxins, such as beta 2 toxin (CPB2, *cpb*2 gene) and perfringolysin O (PFO or theta toxin, *pfo* gene), can act synergistically with extracellular toxins to cause the histotoxic pathogenesis of this organism and promote the progression of illness [3].

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C. perfringens has been estimated to be the second main cause of foodborne outbreaks (FBO) bacterial disease in the United States during 2019 year (almost 1 million cases every year) (https://www.cdc.gov/clostridium-perfringens/about/index.html) and the fourth in Europe [2]. It is also responsible for 5-20% of antibiotics-associated diarrhea and non-FBO diarrhea [1–3]. There is poor representation of isolated *C. perfringens* genomes in developing countries [2]. However, when tracing a local event in Spain, the same limitation in the dataset was observed. The present study explores the genomic features of *C. perfringens* strains isolated from a major FBO.

Materials and methods

Public health investigation of a foodborne outbreak (FBO)

An on-site epidemiological investigation was conducted by the regional Epidemiological Surveillance Service at a school in the Málaga province (36°43′12″N, 4°25′13″W, Andalusia, Spain). The investigation focused on diners, food served and school characteristics, covering approximately 800 people (students, teachers and religious community members). The case definition were individuals who regularly used the school cafeteria and developed a gastroenterological condition (diarrhea occurring more than once a day) between October 18, 2023 and October 19, 2023 (onset date of symptoms for the first case and for the last case). Secondary cases were not confirmed. Non-random systematic sampling for interviews ensured representation across all school levels [6]. Variables studied included age, sex, class/course, cafeteria use per day (October 16, 17 and 18), foods consumed, symptoms onset and evolution. Clinical stool samples from cases and food handlers, as well as food samples, were collected. A logistic regression model was applied, with three days of lunch included as categorical variables to obtain adjusted odds ratios (ORs) [5].

Microbiological findings in the clinical samples

Microbiological investigations were conducted on six patients with diarrhea, using stool culture analysis for entero-pathogenic bacteria, the FilmArray Gastrointestinal panel (BioMerieux) and immunochromatogenic techniques. *C. perfringens* growth was initially detected in cultures from five patients using MALDI-TOFF - MS (Bruker Biotyper, Billerica, MA, USA). DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Strain identification was confirmed by 16S rRNA gene sequence analysis [6] using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). PCR screening for major toxins was performed [7,8].

Genome assembly and annotation

Paired-end libraries were prepared using the Nextera-XT DNA Library Preparation Kit (Illumina 1.9), and sequencing was performed using the Illumina NovaSeq platform. FastQC v.0.11.8 software was used to read quality metrics; Trimmomatic v. 0.33 to remove adapter contamination and trim low-quality regions (http://www.usadellab.org/cms/?page=trimmomatic) [9]; Kmerfinder v. 3.0 was used for species confirmation and contamination detection (https://bitbucket.org/genomicepidemiology/kmerfinder/src) [10]; Unicycler v.0.4.615 [11], https://github.com/rrwick/Unicycler) and Quast v. 4.1 were used for assembly and quality control (https://github.com/ablab/quast) [12]; and Prokka v. 1.12 for genome annotation (https://github.com/tseemann/prokka, accessed on December 2023) [13].

Species assignment and multi-locus sequence typing

Species identification was conducted using average nucleotide identity (ANI) (https://www.ezbiocloud.net/tools/ani) [14], digital DDH by genome BLAST distance (GBDP), and the percentage difference in G+C content through the Type Strain Genome Server or TYGS (https://tygs.dsmz.de/) [15]. The classical multi-locus sequence typing scheme (MLST) [16] and the core genome cgMLST scheme (with 1431 highly conserved core genes) [17] were applied by querying the genomes against the *C. perfringens* PubMLST database (https://pubmlst.org/organisms/clostridium-perfringens, accessed on December 27, 2023). The phylogenetic analysis was visualized using the iTOL tool (https://itol.embl.de/).

Antimicrobial susceptibility and resistome

The antimicrobial susceptibility profile was determined using the E-test as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_14.0_Breakpoint_Tables.pdf), and categories were established following EUCAST and Clinical and Laboratory Standards Institute (CLSI) criteria [18,19]. The resistome analysis was conducted using the resFinder v.4.4.2 software (http://genepi.food.dtu.dk/resfinder Center for Genomic Epidemiology), the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca/analyze/rgi), and the KOALA for KEGG Orthology database (https://www.kegg.jp/ghostkoala, accessed on May 2024) [20].

Detection of virulence determinants, plasmids and prophages

The protein sequences of the defined virulence factors of *C. perfringens* [21] were queried against assembled genomes using the BLASTp program (minimum identity of 80%, minimum coverage of 60%), including ν-toxin (CadA, ABG84396.1) and urease (ureABC, CAA71383-5). Plasmids were detected by querying the Plasmid database PLSDB v. 2024_05_31_v2 (https://ccb-microbe.cs.uni-saarland.de/plsdb/plasmids, accessed on December 2024), with the options: mash screen, maximum P-value default of 0.1, minimal identity of 0.99, and the winner-takes-all strategy [22]. Prophages were identified using the PHASTEST (PHAge Search Tool with Enhanced Sequence Translation) webserver (https://phastest.ca/, accessed on December 2024) [23].

Statistical analyses

Statistical descriptions and subsequent analyses were performed based on the phone interviews, with a case-control design to calculate (ORs) as measures of association to confirm the epidemiological exposure. Crude and adjusted ORs were estimated using logistic regression, with potential exposure days as covariates. Statistical significance was set at $P < 0.05$. Network analysis was conducted using Rstudio with R version R4.4.1 2024.04.2. The informed consent requirement was waived.

Results

FBO public health investigation

A total of 511 subjects (495 pupils, 16 teachers) were affected out of an estimated 822 exposed individuals, resulting in an attack rate of 62.17% (Table 1) in the period of the study (16-18 October, 2023). A total of 51 controls (potentially exposed healthy individuals or their guardians) and 99 cases (potentially exposed ill individuals) were interviewed by phone. Women represented 62% of

Table 1
Attack rates of *Clostridium perfringens* among students and school staff for the onset day for symptoms (III, October 18, 2023).

Category	Cases (n)	Exposed individuals (n)	Attack rates
Students	495	773	64.04%
School staff	16	49	32.65&
Total	511	822	62.17%

the interviewed cases. Among interviewed cases, diarrhea affected 84.85% of cases, while 82.83% experienced abdominal pain. Cases with both symptoms were 71.72%, and with either diarrhea or abdominal pain were 100%. The proportion of cases with fever or vomiting were of 4.04% and 3.03%, respectively. A "point source" pattern curve, characteristic of FBO, was observed, peaking at 9:00 PM on day III. All cases were self-limiting with no need of medical treatment. Among the interviewed cases, six were asked to provide a stool sample. Secondary cases, defined as those who would have been infected by one or more primary cases through person-to-person transmission, were not confirmed either by interviews or by notifications from Health Services or School Institutions.

Potential exposure occurred during lunch on the day of the symptom's onset and the two previous days (coded as days I, II and III, with the latter being the onset day). Other possible exposure periods were ruled out based on person-place-time frameworks. Adjusted ORs for days I, II and III were, respectively, 0.61, 1.49 and 81.81. Adjusted OR for day III was statistically significant, with a P-value <0.001 (Wald test) (Table 2) [5]. In parallel, the Regional Department of Health and Consumer Affairs of Andalusia conducted a risk-based inspection to review the chains and circuits of food acquisition, storage and handling that could potentially pose health risks [24]. Food handling deficiencies were detected, specifically during the preparation, storage, and serving of the Bolognese sauce in the school lunch on the day of the onset of symptoms. This pointed to the meat in the sauce as the likely source. Three healthy people from kitchen staff were asked to provide a stool sample, giving negative results.

Species assignment and multi-locus sequence typing analysis

The strains were identified as *C. perfringens* by 16S rDNA gene analysis, which revealed 100% identity with the type strain ATCC 13124. The epidemiological characteristics of patients are compiled in Table S1. PCR screening confirmed that the strains were positive for the *cpa, cpe*, and *cpb*2 genes. Assembly statistics of the genomes are compiled in Supplemental Table S2. Species assignment was confirmed by the genome-based taxonomic coefficients (ANI ≥95%, DDH ≥70%, G+C difference <1%) [14,15] between the genomes of the isolated strains and the reference genome of the strain FDAARGOS_903 (GCF_016027375.1), which corresponds to the type strain ATCC 13124. The strains were clustered into two sets: Set A (CNM20231135, 20231136 and 20231138) and set B (CNM20231137 and CNM20231139), showing an ANI value of ≥99.9 within each set and ≤98.4 between them (Supplemental Table S3). Similarities and differences in the genome sequences were analyzed using BLAST with the Blast Ring Image Generator (BRIG) [25], as shown in Figure 1. Two different sequence-types (STs) and *core genome* sequence-types (cgSTs) were identified: the new ST-804/cgST-804 (with the description of the novel alelle *pgk*-82) for the set A strains, and the ST-5/cgST-801 for the set B strains. The PubMLST *C. perfringens* database includes 517 genomes, including the strains studied [17]. The overall composition of the cgST for 158 genomes of strains with enterotoxin production (CPE-positive) entered into this database showed that the genomes of each set were not closely related. The same lack of similarity was observed when the 42 available CPB2 toxin-positive genomes were analyzed (Figure 2). Less than 6% of the genomes introduced into the PubMLST database were positive for both toxins (n = 28), including these FBO strains.

Antimicrobial susceptibility and resistome

The strains were susceptible to benzylpenicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, imipenem, meropenem, metronidazole, vancomycin and clindamycin, according to the interpretative categories of EUCAST for *C. perfringens* [18]. Using the CLSI MIC breakpoints for anaerobes (Table 2) [19], the strains were susceptible to moxifloxacin and exhibited intermediate susceptibility to tetracycline. The criteria for erythromycin (<2 mg/L), fidaxomicyn (0.5 mg/L), and linezolid (<1 mg/L) are not available. The following antimicrobial resistant traits were detected in all strains: class A beta-lactamase-related serine hydrolase CARB-6; tetracycline efflux MFS transporter TetA(P) (or Tet40); tetracycline resistance efflux pump TetB(P) (or Tet38); ABC-F type ribosomal protection protein CplR (affecting lincosamides); bifunctional lysylphosphatidylglycerol flippase/synthetase MprF; and MATE family efflux transporter MepA. Regarding beta-lactamase CARB-6, two alleles were identified. One allele was shared by set A strains with two amino acid changes (253T-A and 257N-D). The other allele was found in set B strains and exhibited full identity with the corresponding gene in the reference genome of the strain FDAARGOS 903.

Virulome

The following *C. perfringens* virulence traits were detected using the BLASTp tool: alpha-toxin or phospholipase C (encoded by the *cpa* or *plc* gene), enterotoxin (*cpe*), spore-forming cytolytic toxin beta2-(*cpb*2), pore-forming toxin perfringolysin (*pfo*A), collagenase A (*col*A), hyaluronidase (*nag HIJKL*), sialidases (*nan*I, *nan*H, *nan*J), hemolysin III (*hly*III), hyaluronoglucosaminidase (*hy*A), enterotoxin A/B/D (*ent*A, *ent*B, *ent*D), cadmium-translocating P-type ATPase and clostripain (*ccp* or *clo*SI). However, the following toxins were absent: beta-toxin (encoded by the beta-toxin *cpb* genes), epsilon-toxin (*etx*), iota-toxin (*iap/ibp*), lambda-toxin (*lam*), necrotic enteritis toxins or pore-forming leucocidin/hemolysin (*net*BEFG), binary enterotoxin (*bec*AB), large cytotoxin (*tpe*L), delta-toxin (*cpd*), alveolysin (*alv*), and urease (*ure*ABC). Therefore, according to the toxin-based scheme of Rood *et al.* [3], all the strains of this FBO were classified as type F toxins (*cpa*+, *cpb*-,*etx*-,*iA*-, *cpe*+, *net*B-) and, using the modification of Matsuda *et al.* [26], as type H2 (*cpb2*+, *bec/cpile*-). Following Mahamat Abdelrahim *et al.* [27], they exhibited the virulence profile II (*cpb*2+, *ia*-, *ib*-, *cpe*+, *pfo*A+, i*am*-, *nag*H+ *nan*I+ *nan*J+). Using the CPA/PLC typing scheme [26,28], two variants were identified for the major toxin: set A strains showed a 363P-S change (new type), and set B strains showed 13T-A change (Type Ia), compared to those defined for strain 13 (Type F or first type).

Regarding enterotoxin CPE, whether chromosomal or plasmid encoded, a unique sequence was observed in all strains, showing full identity with strain CP396 (MH900564) [26], which contains *cpe* in the plasmid type pCPF4969. In the studied genomes, the *cpe* gene is flanked by the *dcm* gene and the insertion sequences from the IS200/IS605 family transposase (IS1469, IS1470), as occurs when *cpe* is plasmid-located [29]. In addition, the nucleotide sequence of cytotoxin CPB2 in the intestinal cells was identical among the studied strains, and strains CP396 and CPM 77b (GCF_020138375.1) were found to belong to cluster I [26] and located in the *cbp*2 plasmid.

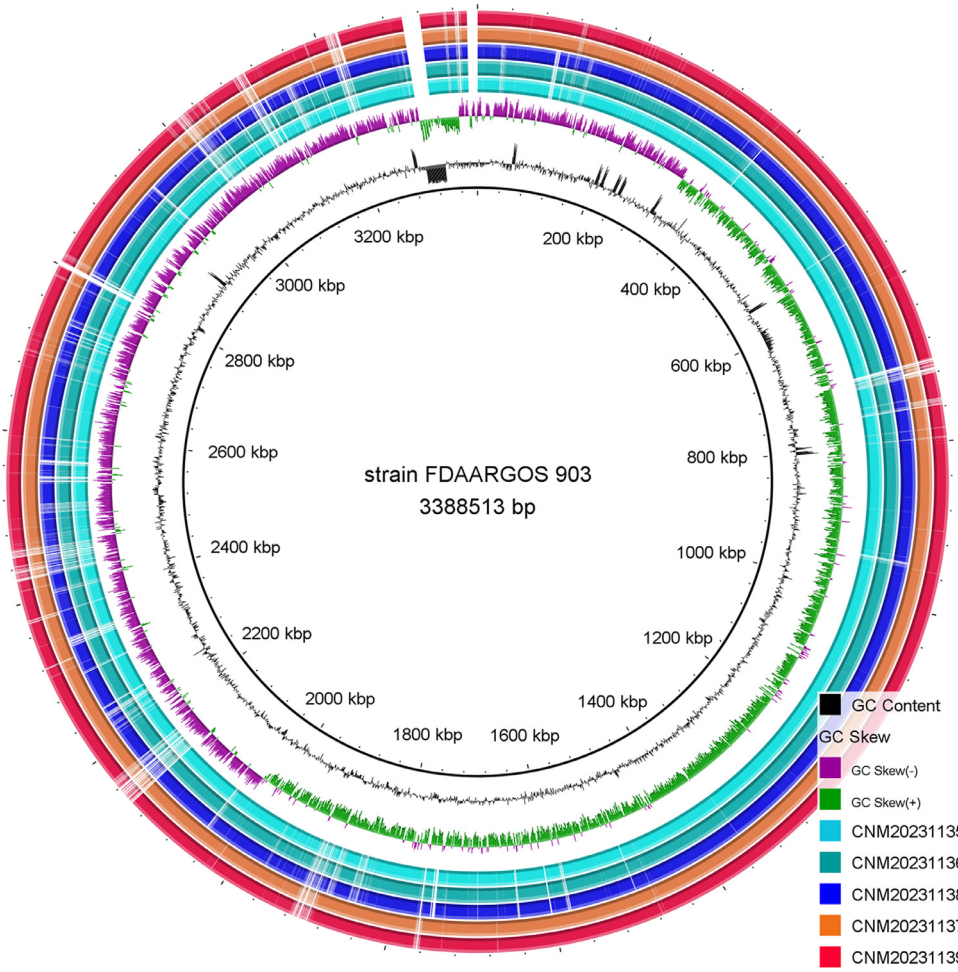

Figure 1. BLAST analysis of *C. perfringens* genome sequences. Genomes were compared pairwise using BLAST and visualized with BRIG against the reference genome FDAARGOS903. Genomes shown include (from inner to outer ring) strains CNM20231135, CNM20231136 and CNM20231138 (set A), and strains CNM20231137 and CNM20231139 (set B) (see Methods for genome accession numbers). The central rings (green and purple) represent the GC skew, calculated for the reference genome of the FDAARGOS903 strain. Genomic regions with less than 80% nucleotide identity compared to the reference sequence are shown with white gaps. BLAST, Basic Local Alignment Search Tool; BRIG, Blast Ring Image Generator.

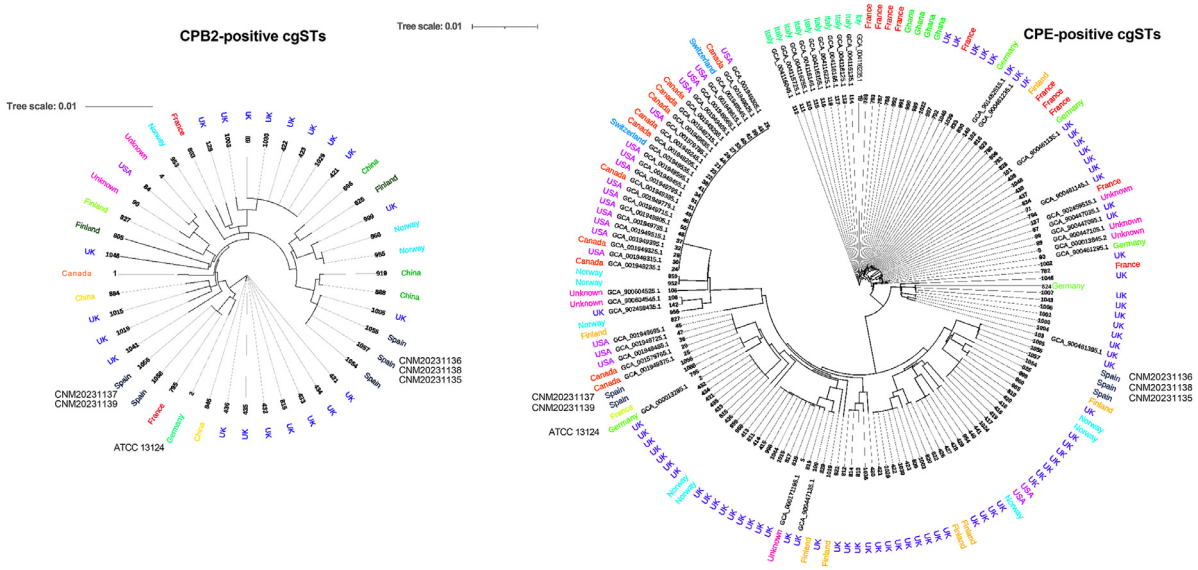

Figure 2. Core genomic relationships of *C. perfringens cpe*-positive strains (n = 158, on the right) and *cpb2*-positive strains (n = 42, on the left) generated using a minimum spanning tree and visualized with iTol from the available genomes on the pubMLST database (https://pubmlst.org/bigsdb?db=pubmlst_cperfringens_isolates&page=plugin&name=ITOL, 1431 core genes). The genome of the ATCC13124 type strain was included.

Table 2
Menu items and adjusted odds ratio (OR) for *C. perfringens* infection among students and school staff for the onset day and the two previous days.

Date	Food item	Regression coefficient[a]	Adjusted OR	P-value[b]
I (Oct 16, Mon) Lunch	lentils with chorizo vegetable stir-fry with chicken strips whole wheat bread and fruit	−0.5	0.61	0.68
II (Oct 17, Tue) Lunch	rice casserole with squid and prawns baked fish with vegetables whole wheat bread and fruit	0.4	1.49	0.5
III (Oct 18, Wed) Lunch	caesar salad bolognese macaroni with cheese[c] macaroni with tomato and tuna whole wheat bread and fruit	4.40	81.81	<0.001

[a] regression coefficient constant value was of −3.37.
[b] Wald test.
[c] when bolognese macaroni with cheese ran out, other batch of macaroni with tomato and tuna was made, and eaten by some students, teachers, and kitchen staff.

Plasmids

In the studied strains, three different plasmids were identified, exhibiting up to 99.99% identity with those previously described [30]: the mobilizable plasmid pCPCPI18-1_1 carrying the *cpe* gene (GenBank accession no. NZ_CP075982.1, size of 70.5 kb, GC content 0.27%, linear topology, replicon type rep_cluster_1519) and the mobilizable plasmid pCPCPI18-1_2 carrying the *cpb*2 gene (NZ_CP075983.1, 53.7 kb, 0.25% GC, linear, rep_cluster_1713) were both identified in *C. perfringens* strain CPI 18-1b (GCA_020138795.1); the non-mobilizable plasmid pCPCPLi6-1_4 (NZ_CP075916.1, 13.0 kb, 0.24% GC, linear, rep_cluster_427) without virulence genes was identified in *C. perfringens* strain CPLi 6-1 [30]. The genetic organization of the *cpe* and *cpb*2 loci were quite similar among the strains in both sets. The plasmid-*cpe* locus of the strains corresponds to the pCPF4969-like *cpe*-carrying plasmid (AB236336) type, which includes the *dcm* gene and two distinctive insertion sequences (IS1469 and IS1470) with a region encoding the *tcp* (transfer of clostridial plasmids) with putative bacteriocins and a VirR/VirS two-component regulatory system [21,29]. The *tcp* locus has been regarded as a reasonable predictor of their conjugative potential, enhancing virulence diversity. The *tcp* locus was also present in other previously described plasmids, including pJIR3844 (JN689217), which carries the *cpb*2 gene, and pCW3 (DQ366035) and pJIR3537 (JN689220), which carry the *tet* gene. In the studied genomes, plasmids carrying the *tet* gene were not detected using PLSDB; however, their presence is inferred by Proksee (blast tool, https://proksee.ca) (Figure 3). The composition of contigs that include *cpe* and *cpb*2 plasmids is shown in Figure 4.

Prophages

The genome of *C. perfringens* is rich in phage elements, which have been reported to play a significant role in shaping gut bacterial composition. Four intact prophage regions have been observed in all strains (Table S4 and Figure 5), except for strain CNM20231136. The episomal phage ϕSM101 is the unique phage the two sets have in common, while the typical *C. perfringens* phages, ϕ3626 and ϕCP51, were only detected in set A and in set B, respectively. Other phages described in other clostridia or gram-positive organisms were also observed: in set A, ϕCTC2B and ϕCT19406B (only detected in strain CNM20231136) of *Clostridium tetani* and ΦFL4A of *Enterococcus faecalis*; in set B, ΦCTP1 of *C. tetani* and ϕCD111 of *Clostridioides difficile*. The presence of prophages and, more notably, the absence of CRISPR defense systems (as observed in these strains) in more than 70% of the *C. perfringens* strains has been indicative of significant horizontal gene transfer events [31].

Discussion

The presence of *C. perfringens* in diverse environmental niches (soils, food and sewage) and in the gastrointestinal microbiota of sick and healthy humans and animals promotes its global spread, making it one of the main foodborne pathogens of humans [1–3]. The strains of the toxinotype F produce highly resistant spores that can germinate very quickly in improperly stored or undercooked food, leading to a substantial proliferation of vegetative cells. Large pieces of meat are often associated with FBO via cross-contamination from food or contaminated surfaces during the slaughter process [4,31]; however, vegetable meals can also be a source [27]. The epidemiological and microbiological investigation of an FBO that affected 526 individuals at a school in Málaga (Andalusia, Spain) is discussed here.

The possible exposure event was the meal on the day of onset of cases (OR 81.81, P <0.05), and the probable source was the meat in the Bolognese sauce. When the Bolognese sauce was finished, it was replaced by tuna and tomato sauce. People who consumed macaroni with tuna and tomato did not become ill. The FBO exhibited a typical unimodal epidemic curve, with symptoms appearing on day III.

The low rate of microbiological confirmation among the epidemiologically linked cases is the main limitation of this study is. Despite a high attack rate of 62%, *C. perfringens* could only be isolated from five patients. This discrepancy is attributed to factors inherent to the microbiology and epidemiology of *C. perfringens* food poisoning such as brief clinical course and delayed sampling. Future studies should prioritize early sample collection to improve the isolation rate.

Toxinotype F is the main cause of FBO, but it is also involved in cases of antibiotics-associated diarrhea and non-foodborne enteritis [4,27]. The lysing cells of toxinotype F sporulate and release CPE to the intestinal lumen, causing typical symptoms, such as diarrhea and abdominal cramps within 12-24 hours, that resolve without complications within one day, as was the case for the individuals implicated in the studied FBO. However, under specific circumstances (previous constipation or fecal impaction, use of psychoactive drugs, or age), a fatal course of the disease can occur. It is important to note that the course of food poisoning illnesses differs from the severe course of the antibiotics-associated diarrhea caused by this pathogen, which can lead to intestinal inflammation and necrosis and may last several weeks [31]. The brief and non-severe clinical course of FBOs caused by *C. perfringens* often underestimates the significance of these events.

The study of the genomic features of the strains involved in this FBO revealed the participation of two clones of the newly designed toxinotype F [3,4], suggesting a multiple contamination sources in the same outbreak. This clonal heterogeneity is unusual for a sim-

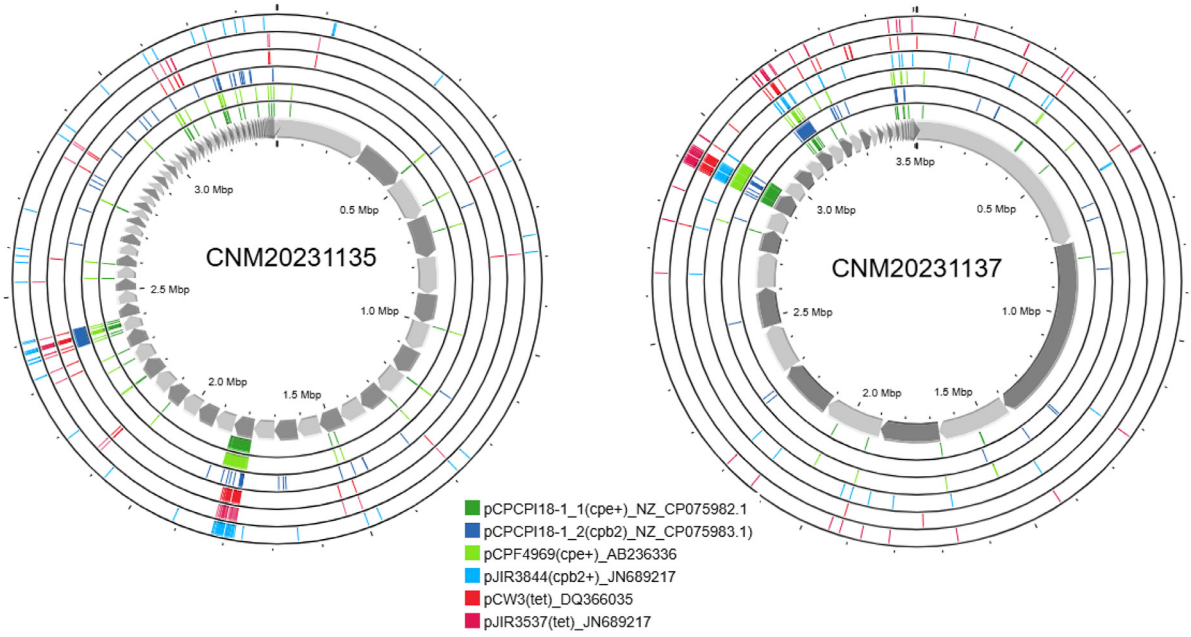

Figure 3. Genomic location of plasmids pCPCPI18-1.1 and pCPF4969 carrying the *cpe* gene, pCPCPI18-1.2 and pJIR3844 carrying the *cpb2* gene, and pCW3 and pJIR3537 carrying the *tet* gene using Proksee (blast tool, https://proksee.ca) for strains CNM20231135 and CNM20231137.

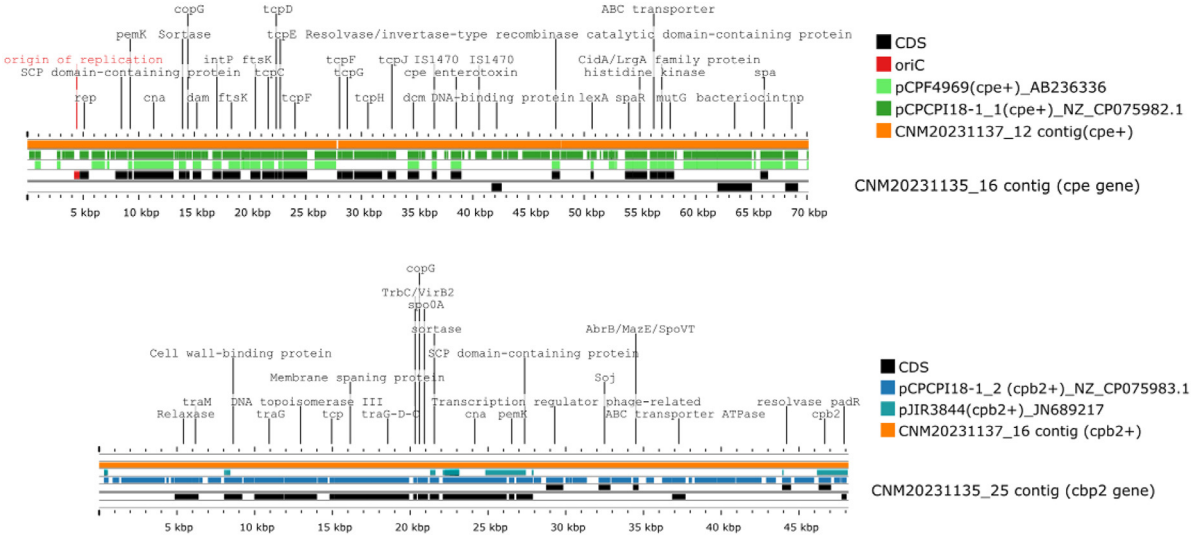

Figure 4. Composition of plasmids containing the *cpe* (up) and *cpb2* (down) genes located in contigs 16 and 25, respectively, of the genome of the set A strain CNM20231135 using Proksee (blast tool, https://proksee.ca/). The alignment is shown with respect to the set B strain CNM20231137, and analog plasmids are included.

ple, single point-source outbreak, as evidenced by the presence of two distinct genomic clusters. Two possibilities are considered: the point source was a complex contamination vehicle that harbored two different *C. perfringens* strains; or there were two separate but related contamination events. Despite the genetic findings, the epidemiological data (common exposure, high attack rate) remains the strongest evidence linking the illnesses to a single food-borne outbreak.

In comparative genome studies, the toxinotype A (only alpha-toxin positive), followed by toxinotype F (alpha and CPE- positive), have been described as the most frequent toxinotypes in human isolates (60% and 32%, respectively), with variations based on regional differences and sources. Other clinically relevant accessory toxins such as sialidase (*nan*H), clostripain (*ccp*) and collagenase A (*col*A) have been identified as the main virulence genes (≥98%) [2]. In the studied strains, these last genes are also present, along with *cpb2*, perfringolysin (*pfo*A), hyaluronidase (*nag*H), sialidases (*nan*IJ), hemolysin (*hly*III), enterotoxin-related virulence factor (*ent*B) and other minor virulence genes. The PFO toxin acts synergistically with alpha-toxin, and sialidase NanI is linked to enhanced intestinal attachment and plays an accessory role in enhancing CPE. This virulence profile differs from the virulome identified in antibiotics-associated diarrhea strains (n = 36), where *cpb2* and *pfo*A genes are not detected and the *cpe* gene is detected at a low rate (6%) [32].

The toxins and virulence genes of *C. perfringens* can either be encoded chromosomally (CPA, PFO, kappa-toxin, collagenase, hyaluronidases, sialidases and clostripain), plasmid-encoded (CPB, CPB2, ETX, ITX, NETB, large cytotoxin tpeL, hemolysin delta-toxin, and matallo-protease lambda-toxin), or both chromosomally and plasmid encoded, as in enterotoxin CPE [29]. Small plasmids (10 kb) and large conjugative plasmids (45-140 kb) carrying up to 16

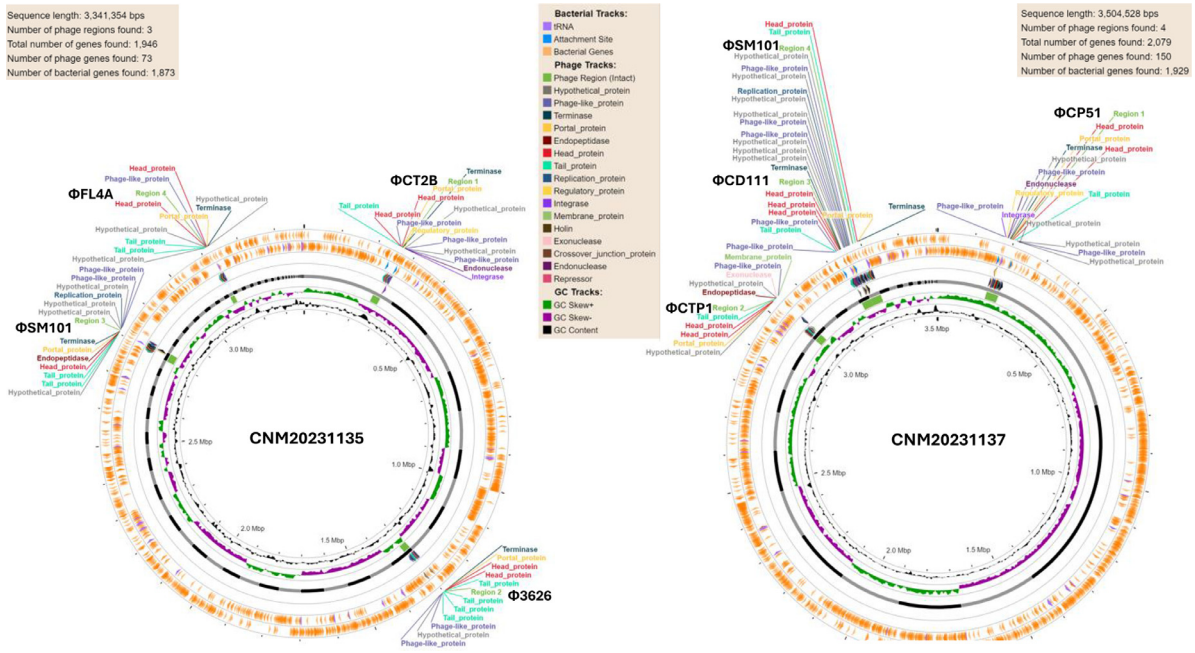

Figure 5. Composition and location of the detected phages in the FBO *C. perfringens* strains CNM20231135 and CNM20231137, identified using the PHASTEST server. FBO, food-borne outbreaks.

toxin genes and antimicrobial resistance genes have been identified at low copy numbers. Some strains carry up to three closely related toxin plasmids, and a single plasmid can contain up to three distinct toxin genes. But, this figure has recently increased to ten plasmids per strain [21]. The presence of plasmids with toxins and antibiotic resistance genes is associated with the ability to survive in multiple and diverse environments such as soil and the gastrointestinal tract, which impacts the disease outcomes [2].

Enterotoxin CPE, whether chromosomally or plasmid-encoded, is known to be a key factor in food poisoning and non-foodborne gastroenteritis. Nearly 80% of type F food poisoning strains carry the chromosomal c-*cpe* gene, rather than the plasmid-borne *p-cpe* gene [2,32,33]. Spores from strains with the *c-cpe* gene can exhibit higher temperature resistance (for *c-cpe* group 1, but not group 2) [29,30], allowing them to better survive in undercooked food. Most of these strains lack perfringolysin, hyaluronidases and sialidases, which are considered important host-associated genes for colonization [34]. In contrast, antibiotics-associated diarrhea and non-foodborne diarrhea strains usually have a plasmid-borne *cpe* gene closely associated with either downstream IS1151 or defective IS1470-like sequences [35], along with perfringolysin, hyaluronidases and sialidases. These strains have more sensitive spores [29]. It has been observed that in the United Kingdon plasmid-*cpe* (due to pCPF4969 or pCPF5603) is a relatively common occurrence in FBO [36]. Core genome phylogenomic inference has shown that *c-cpe* strains have independently evolved from *p-cpe* or *cpe*-negative strains [27]. All the Spanish FBO strains exhibited an atypical infection profile linked to food and an increased virulence potential, with the *cpe* gene located on a pCPF4969 plasmid type, similarly to those described previously and found in lineage V (*21,29,30*), along with CPB2 and PFO. As described for *c-cpe* [2,30], this *p-cpe* has a propensity to thrive in food environments and contributes to the development of major FBOs, despite their sensitive spores.

In previous studies, the incidence of *cpb*2-positive strains was low and belonged to dispersed clonal lineages, exhibiting a different distribution pattern than that of *cpe*-positive strains. The *cpe*-positive strains tend to be carried by livestock, while the *cpb*2-positive strains have been identified in cases of diarrhea, in healthy individuals and in children with autism. An epidemiological study of stool samples from individuals with and without gastrointestinal symptoms revealed that *cpb*2 gene detection was associated with the presence of diabetes or autoimmune diseases, suggesting a possible interaction between this toxin and the immune system [37].

The location of major toxins, such as CPE and CPB2, can influence the outcomes of a disease. Chromosomal variants may be lost through mutations or deletions, leading to shorter illness due to low colonization levels and spreading. In contrast, the plasmid-encoded toxins can be transferred by conjugation to strains of *C. perfringens* in the intestinal microbiota, promoting enhanced colonization and virulence [38,39] and resulting in more severe and prolonged illnesses, as seen in antibiotics-associated diarrhea.

Effective control of *C. perfringens* FBO diseases requires improved food handling procedures within large-scale user facilities and genomic analysis at the local and global levels, as shown in the present study. This is the first genomic surveillance of the virulence of *C. perfringens* strains causing the largest FBO in Spain by this pathogen.

Ethics approval

The clinical samples were taken as part of standard patient care and also for the purpose of this study. This study was focused on bacteria and no identifiable human data were used. Therefore, ethical approval was exempted. For epidemiological survey, verbal consent was obtained.

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Data availability

Raw epidemiological data for this study are not publicly available due to legal and ethical constraints related to patient privacy and institutional protocols of the Andalusian Health Service (SAS). The data, originally collected for clinical and public health purposes, was accessed by the authors from the Prevention, Promotion, and Surveillance Unit, Málaga Health District, under a non-disclosure agreement. Only the processed and anonymized datasets, as used in the official technical reports, were utilized for this article.

The Whole Genome Shotgun project PRJNA1057079 was deposited at DDBJ/ENA/GenBank under accessions JAYGGH000000000, JAYGGI000000000, JAYGGJ000000000, JAYGGK000000000 and JAYGGL000000000, included in BioProject Accession PRJNA1057079 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1057079).

Author contributions

Sylvia Valdezate: Study design, Data collection, Data analysis, Writing. **María J. Medina-Pascual**: Study Data collection, Data analysis. **César Rodríguez**: Study design, Data collection, Data analysis, Writing. **María Pérez-Vázquez**: Data analysis, Writing. **Mónica Valiente**: Data collection. **José Serrano**: Data collection. **Marta Rodréguez**: Data collection. **Inmaculada De Toro**: Data collection. **Pilar Villalón**: Study design Writing.

Declaration of competing interest

The authors have no competing interests to declare

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2025.108179.

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