International Journal of Food Microbiology 412 (2024) 110554

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Yersinia enterocolitica biovar 1A: An underappreciated potential pathogen in the food chain

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ARTICLE INFO

Keywords:
Yersinia enterocolitica
Whole genome sequencing
Food

ABSTRACT

Yersinia enterocolitica is an underreported cause of foodborne gastroenteritis. Little is known of the diversity of *Y. enterocolitica* isolated from food and which food commodities contribute to human disease. In this study, *Y. enterocolitica* was isolated from 37/50 raw chicken, 8/10 pork, 8/10 salmon and 1/10 leafy green samples collected at retail in the UK. Up to 10 presumptive *Y. enterocolitica* isolates per positive sample underwent whole genome sequencing (WGS) and were compared with publicly available genomes. In total, 207 *Y. enterocolitica* isolates were analyzed and belonged to 38 sequence types (STs). Up to five STs of *Y. enterocolitica* were isolated from individual food samples and isolates belonging to the same sample and ST differed by 0–74 single nucleotide polymorphisms (SNPs). Biotype was predicted for 205 (99 %) genomes that all belonged to biotype 1A, previously described as non-pathogenic. However, around half (51 %) of food samples contained isolates belonging to the same ST as previously isolated from UK human cases. The closest human-derived isolates shared between 17 and 7978 single nucleotide polymorphisms (SNPs) with the food isolates. Extensive food surveillance is required to determine what food sources are responsible for *Y. enterocolitica* infections and to re-examine the role of biotype 1A as a human pathogen.

1. Introduction

Yersiniosis is an infectious disease caused by bacteria belonging to the *Yersinia* genus. In 2021, yersiniosis was the third most commonly reported zoonosis in the European Union, with the primary agent being *Yersinia enterocolitica* (EFSA and ECDC, 2022). Primary *Y. enterocolitica* infections affect the gastrointestinal tract, causing symptoms of diarrhea, fever, stomach cramps, vomiting and blood in stools (Huovinen et al., 2010). Whilst *Y. enterocolitica* infections are generally self-limiting, secondary complications include sepsis (Thwaites and Woods, 2017), focal infections (Rodio et al., 2018), ileitis (Bailly et al., 1991), appendicitis (Fukushima et al., 1981) and reactive arthritis (Fendler et al., 2001).

Yersiniosis is an under-ascertained foodborne disease. In the county of Hampshire in the South East of England, the Fast-Track Diagnostics Bacterial gastroenteritis panel FTD-14.1–64 PCR method was applied to all diarrheic stool samples to improve the detection of *Yersinia* from fecal samples and resulted in an almost 100-fold increase in the number of *Yersinia* detected over traditional culturing, with approximately 20 % of isolates belonging to biotype 1A (Clarke et al., 2020). Prior to the introduction of the PCR method, the laboratory followed the UK Standards for Microbiology Investigations and only cultured fecal samples for *Y. enterocolitica* when the patient had symptoms characteristic of an invasive *Y. enterocolitica* infection, *e.g.*, appendicitis, mesenteric lymphadenitis, terminal ileitis or reactive arthritis (Public Health England, 2020). Strains of *Y. enterocolitica* are zoonotic and foodborne, but

Abbreviations: AMR, antimicrobial resistance; BBSRC, Biotechnology and Biological Sciences Research Council; BWA, Burrows-Wheeler aligner; CIN, cystine-lactose-electrolyte deficient agar; FSA, Food Standards Agency; ITC, irgasan-ticarcillin-potassium chlorate; MLST, multi-locus sequence typing; PSB, peptone sorbitol and bile salts; QAC, quaternary ammonium compounds; QRDR, quinolone resistance-determining region; SRA, Sequence Read Archive; SNP, single nucleotide polymorphism; ST, sequence type; TPW, tris-buffered peptone water; WGS, whole genome sequencing; *Y. enterocolitica*, *Yersinia enterocolitica*.

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https://doi.org/10.1016/j.ijfoodmicro.2023.110554
Received 8 September 2023; Received in revised form 5 December 2023; Accepted 21 December 2023
Available online 26 December 2023

despite the perceived importance of this transmission pathway, there are currently no routine surveillance strategies for testing food in England.

Y. enterocolitica has traditionally been associated with pork; the bacterium can be found throughout the pork production chain (Martins et al., 2022; Terentjeva et al., 2022), and consumption of undercooked meat is a risk factor for *Y. enterocolitica* infection (Tauxe et al., 1987). However, factors not directly associated with pork have also been associated with *Y. enterocolitica*, such as consuming unpasteurized milk, eating outside the home and travel (Huovinen et al., 2010). *Y. enterocolitica* has also been isolated from other food-producing animals, such as chicken, cattle and sheep, and other food products, such as leafy greens (Fearnley et al., 2005; MacDonald et al., 2012; Momtaz et al., 2013). Few studies have investigated the diversity of *Y. enterocolitica* on retail food (Davies et al., 2001) and less still if the *Y. enterocolitica* present on food is clinically significant (Esnault et al., 2013).

Y. enterocolitica can be divided into six biotypes using biochemical testing that vary in pathogenicity and ability to cause disease. However, whole genome sequencing (WGS) has allowed a better understanding of the phylogenetic structure of this species (Hall et al., 2015), and phylogroups have been proposed to replace the traditional biotypes and can be distinguished based on O-antigen specific genes (Reuter et al., 2015). Historically, biotype 1A (phylogroup 1) was considered non-pathogenic (Sihvonen et al., 2012), biotype 1B (phylogroup 2) as highly pathogenic (Carniel, 2001) and biotypes 2–5 as low-to-moderate pathogenic strains (Valentin-Weigand et al., 2014). Isolates belonging to biotypes 2–5 comprise phylogroups 3–6, but some isolates that are classified as the same biotype belong to different phylogroups (*e.g.*, biotype 3 consists of isolates belonging to phylogroups 4 and 5) and *vice versa* for this group (*e.g.*, phylogroup 5 consists of isolates belonging to biotypes 2 and 3) (Reuter et al., 2014).

Although the European Centre for Disease Prevention and Control has defined *Y. enterocolitica* biotype 1A as non-pathogenic and not reportable (ECDC, 2016), all biotypes, including 1A, have been isolated from clinical samples (Hunter et al., 2019). Virulence assays using *Galleria mellonella* suggest that all *Y. enterocolitica* are pathogenic (Alenizi et al., 2016). However, *Yersinia* species that are not regarded as human pathogens, such as *Yersinia intermedia* and *Yersinia frederiksenii*, also display virulence in this insect model (Springer et al., 2018), are excreted by livestock (McNally et al., 2004) and can reach humans *via* food (Greenwood and Hooper, 1989). Through high-resolution comparison of *Y. enterocolitica* from clinical cases and food sources, the clinical risk associated with foodborne sources can be evaluated. Whole genome sequencing (WGS) can provide this high-resolution comparison, able to discriminate between *Y. enterocolitica* strains indistinguishable using multi-locus sequence typing (MLST) and multiple-locus variable-number tandem repeat analysis (Inns et al., 2018).

Antimicrobial resistance (AMR) is responsible for an estimated 4.95 million deaths each year (Murray et al., 2022) and food contains a diverse population of AMR determinants (Bloomfield et al., 2023b). Previous studies on *Y. enterocolitica* from frozen food from China found all strains were resistant to two or more antimicrobial classes (Ye et al., 2015), whilst studies on *Y. enterocolitica* on pork found that the levels of AMR correlated with levels of antimicrobial usage for animal production in the country of origin (Koskinen et al., 2022). However, food can be contaminated by a wide range of microorganisms and further research is required to determine how *Y. enterocolitica* contributes to the overall reservoir of AMR genes on food, which could be horizontally transferred to other microorganisms through mobile genetic elements such as plasmids (Karlsson et al., 2021).

In addition to the potential transmission of AMR, plasmids are also important for virulence in *Y. enterocolitica*. The most important plasmid for *Y. enterocolitica* is the temperature sensitive plasmid pYV that contains multiple virulence genes (Rohde et al., 1999). However, there is limited research on the roles of *Y. enterocolitica* plasmids other than pYV.

In a previous study we found evidence of *Y. enterocolitica* in the metagenomes of a wide range of food commodities (Bloomfield et al., 2023b). Other studies have found diverse strains in clusters of human infection, suggesting multiple sources and transmission pathways (Inns et al., 2018). In this study we aimed to determine the diversity of *Y. enterocolitica* on different food commodities and if *Y. enterocolitica* from retail food contributes to human infections or acts as a reservoir of AMR genes. This was achieved by culturing food for *Y. enterocolitica* using multiple methods, sequencing the isolates using WGS, and comparing the genomes of the food-derived isolates to publicly available *Y. enterocolitica* genomes.

2. Material and methods

2.1. Sampling

A convenience sample of 50 chicken, 10 leafy greens, 10 pork and 10 salmon samples collected from retail in Norwich, Norfolk, UK, from 14/03/2021-26/11/2021 were examined for the presence of *Y. enterocolitica*. All samples were fresh/refrigerated, apart from one salmon sample that had previously been frozen. More chicken samples were investigated as a concurrent study on *Campylobacter* in retail chicken was being conducted at the same time. Chicken pieces included skin on and skinless, bone-in and boneless; pork included bone-in and boneless; salmon included skin on and skinless; and leafy greens included whole lettuce, leaves from a single plant species and mixtures of leaves from different plant species. The food samples were cultured for the detection of *Y. enterocolitica* based on the ISO 10273 standard. A tris-buffered peptone water (TPW) method used by the Rare and Imported Pathogens Laboratory for isolating *Y. enterocolitica* from water was also used to detect the targeted bacteria from a subset of the food products collected between 14/03/2021–19/06/2021.

2.2. Methods based on ISO standard

The ISO standard suggests three methods for isolating *Y. enterocolitica*: direct plating, peptone sorbitol and bile salts (PSB) broth (Oxoid, Basingstoke, UK), and irgasan, ticarcillin and potassium chlorate (ITC) broth (Sigma-Aldrich, St Louis, MO, USA). For each sample in this study, 25 g was placed into a FBAG-03 filter blender bag (Corning, New York, NY, USA) and stomached with 225 mL of PSB broth for 60 s. For samples that contained bones, homogenization was performed manually by massaging the stomacher bags for 2 min.

For direct plating, 1 mL of stomached PSB was spread over four cefsulodin, irgasan and novobiocin agar plates (CIN) (Oxoid) plates. CIN plates were incubated at 30 °C for 24 h ± 4 h.

For enrichment, 10 mL of the PSB suspension were transferred into 90 mL of ITC broth. The ITC broth and the remaining PSB suspension were then incubated at 25 °C for 44 h ± 4 h. After incubation, 0.5 mL of each enrichment medium was placed into 4.5 mL of 0.5 % of potassium hydroxide (KOH) solution and mixed for 20 ± 5 s, before a loopful was inoculated onto CIN agar, streaked for single colonies, and incubated at 30 °C for 24 h ± 4 h. KOH treatment inhibits non-*Yersinia* bacteria, increasing *Yersinia* isolation sensitivity (Fukushima, 1985).

From each CIN plate up to four colonies considered to be typical for *Y. enterocolitica* (dark red, bullseye center surrounded by a transparent border) were streaked onto separate CIN plates to allow well isolated colonies to develop. Plates were incubated at 30 °C for 24 h ± 4 h. Isolates from the second CIN plate were streaked onto tryptic soy agar (TSA) (Trafalgar Scientific Ltd., Leicester, UK) to conduct biochemical confirmation. Biochemical tests included using Simmon's citrate agar (Sigma-Aldrich), urea slopes (Oxoid), Triple Sugar Iron (TSI) agar (Sigma-Aldrich) and screening for oxidase production using an oxidase test (Oxoid). From 08/05/2021, rhamnose fermentation broth (rhamnose from Sigma-Aldrich; Phenol red base from Oxoid) was introduced to improve specificity for *Y. enterocolitica*. For citrate, urea, TSI and rhamnose fermentation tests, the media was incubated for 24 h ± 4 h at

30 °C after inoculation. Colonies that displayed a yellow butt and yellow slant on TSI with no gas or hydrogen sulfide, and were oxidase negative, citrate negative, rhamnose negative and urease positive were further analyzed.

2.3. TPW method

For the TPW method, 25 g of each food sample was stomached as above with 100 mL of TPW (Oxoid) for 30 s and incubated at 25 °C for 7 days. Following incubation, KOH treatment was performed as previously described, before a loopful of the TPW suspension was inoculated onto CIN agar plates, streaked for single colonies and incubated for 24 h ± 2 h at 30 °C. From each CIN plate up to five colonies considered to be typical for *Y. enterocolitica* were streaked onto Cystine-Lactose-Electrolyte Deficient Agar (CLED) (Oxoid) and incubated for 24 h ± 2 h at 30 °C. Blue and transparent colonies on CLED were streaked onto TSA and incubated for 24 h ± 2 h at 30 °C. The biochemical tests above were used to identify potential *Y. enterocolitica.*

2.4. Whole genome sequencing

DNA was extracted using the Maxwell® RSC Cultured Cells DNA Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Libraries were created using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, California, USA) and sequenced on a NextSeq 550 System (Illumina) producing 150 base pair (bp) paired-end reads. Raw reads were uploaded to the Sequence Read Archive (SRA) under project PRJNA1003829.

2.5. Genome analysis

Genomic analyses were performed on the Cloud Infrastructure for Microbial Bioinformatics server (Connor et al., 2016). Illumina reads were trimmed using Trimmomatic v0.38 (Bolger et al., 2014) (Appendix A). Trimmed reads were assembled using Spades v3.14.1 (Bankevich et al., 2012) in "careful" mode. The quality of the assemblies was assessed using QUAST v5.0.0 (Gurevich et al., 2013), CheckM v1.0.11 (Parks et al., 2015) and by aligning reads to the assemblies using the Burrows-Wheeler aligner (BWA) v0.7.17 (Li and Durbin, 2009). Assemblies were accepted if they consisted of less than 500 contigs that were over 500 bp, less than 50 duplicate genes and had a mean read depth of the four largest contigs above 30. Centrifuge v1.0.4 (Kim et al., 2016) was performed on trimmed reads to verify them as *Y. enterocolitica*.

EnteroBase (Zhou et al., 2020) was searched for all genomes that were classified as *Y. enterocolitica* (Table B.1). The raw reads of these genomes were downloaded and quality controlled as above.

Prokka v1.14.6 (Seemann, 2014) was used to annotate assemblies. Roary v3.11.2 (Page et al., 2015) was used to cluster annotated assemblies with a 95 % percentage identity and core gene threshold, and form a core gene alignment. RAxML v8.2.12 (Stamatakis, 2014) was used to generate a maximum likelihood tree based on single nucleotide polymorphisms (SNPs) found in this core gene alignment. Some of the EnteroBase genomes had information on the biotype to which they belonged; isolates in the same clade as these genomes were assumed to be the same biotype.

2.6. MLST typing

In silico MLST was performed using MLST v2.16.1 (https://github.com/tseemann/mlst), the genes described by Hall et al. (2015) and the EnteroBase database of known allele profiles. Undefined MLST profiles were classified as novel in this study (Table C.1).

2.7. Genetic elements of interest

ARIBA v2.14.4 (Hunt et al., 2017) was used to identify AMR genes, plasmid replicons and virulence genes using the NCBI (Feldgarden et al., 2019), PlasmidFinder (Carattoli et al., 2014) and VFDB (Chen et al., 2016) databases, respectively, and a 90 % identity cut-off. In addition, a custom database of *gyrA*, *gyrB*, *parC* and *parE* genes from NZ_CP011118 was used to identify point mutations in the quinolone resistance-determining region (QRDR) of *Y. enterocolitica*. ARIBA was also used to predict the *Y. enterocolitica* serotype *in silico* using the database described by Hunter et al. (2019), but with an 80 % identity cut-off.

To determine if *Y. enterocolitica* isolates clustered by the presence of virulence genes, the Jaccard distance was calculated from a presence-absence matrix of virulence genes using the vegan v2.5.6 package (https://github.com/vegandevs/vegan) of R v4.1.2 (R core team, 2019). Non-metric multi-dimensional scaling was performed on the distance matrix to visualize the dissimilarity between genomes and colored by the metadata variables.

2.8. SNP analysis

The *Y. enterocolitica* genome, NZ_HF571988, was used as the reference genome for SNP analysis. Prophage regions were identified in this reference using Phaster (Arndt et al., 2016) and were blocked out from further analysis.

All publicly available *Y. enterocolitica* genomes that belonged to the same sequence type (ST) as those collected in this study were aligned to the reference genome using Snippy v4.6.0 (https://github.com/tseemann/snippy). The full alignments for each ST had recombinant regions identified and removed using Gubbins v2.3.1 (Croucher et al., 2015). The number of core non-recombinant SNPs amongst genomes were then identified.

3. Results

3.1. Isolates

Y. enterocolitica was isolated from 54/80 (68 %) of food samples: 37/50 (74 %) of chicken, 1/10 (10 %) of leafy greens, 8/10 (80 %) of pork and 8/10 (80 %) of salmon samples (Fig. 1). These proportions were significantly different (Fisher's exact test: $p = 0.0007$). From these 80 food samples, 207 *Y. enterocolitica* isolates were recovered and their genomes sequenced.

Initially rhamnose fermentation was not used to identify *Y. enterocolitica*, but only 30 % of sequenced isolates were *Y. enterocolitica*. The introduction of the rhamnose fermentation test on 08/05/2021 resulted in 80 % of sequenced isolates being confirmed *Y. enterocolitica* with WGS. The misidentified isolates were classified as *Yersinia intermedia* ($n = 58$), *Yersinia frederiksenii* ($n = 17$), *Yersinia aleksiciae* (n = 1) and *Rahnella aquatilis* (n = 1).

3.2. MLST

The isolates recovered from food belonged to 38 STs; 16 STs were isolated from more than one sample and 12 STs were isolated from more than one food commodity (Fig. 2). Seventeen STs were only isolated from chicken, one ST was only isolated from leafy greens, three STs were only isolated from pork and five STs were only isolated from salmon. However, only four of these food-commodity specific STs were isolated from more than one sample: three chicken- and one salmon-specific STs.

3.3. Method comparison

Initially four methods were utilized to culture for *Y. enterocolitica* on food: the direct plating, ITC broth enrichment and PSB broth enrichment that were recommended in the ISO standard, along with TPW broth

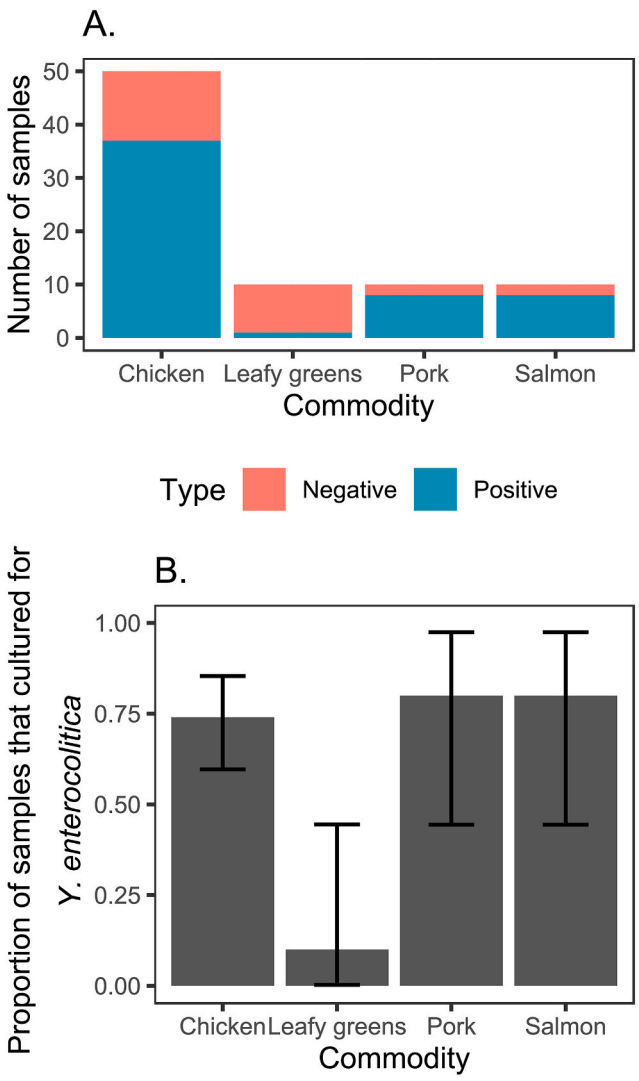

Fig. 1. Number of food samples tested for *Y. enterocolitica* from different food commodities and colored by culture status (A); and the proportion of food samples that cultured for *Y. enterocolitica* for each food commodity with bars representing the 95 % binomial confidence intervals (B).

enrichment. These methods were applied to 35 chicken, five leafy green and five pork samples, identifying 22 STs amongst these samples. Of these STs, one was only identified using direct plating, three only using ITC broth, seven only using PSB broth, two only using TPW broth and nine were identified using multiple methods (Fig. A.1). The TPW broth enrichment method takes seven days, so to speed up the isolation process this method was removed from the sample processing workstream after the first 45 samples, leaving just the methods based on the ISO standard.

All food samples analyzed in this study were cultured for *Y. enterocolitica* using the methods based on the ISO standard (direct plating, ITC broth and PSB broth). These methods identified 38 STs amongst the 80 food samples. Of these STs, five were only identified using direct plating, five were only identified using ITC broth, 13 were only identified using PSB broth, and 15 were identified using multiple methods (Fig. A.2). Of the 23 STs only identified using one method, 20 were only identified in single samples.

3.4. Publicly available genomes

The 207 *Y. enterocolitica* genomes from food were compared with 747 publicly available *Y. enterocolitica* genomes for two purposes: 1) to assist in the prediction of the biotype of the food isolates and 2) to assess if the food isolates had the potential to be clinically significant. The publicly available genomes belonged to isolates collected between 1934 and 2021 from countries around the world, but most commonly from European (28 %) and North American (5 %) countries (Fig. A.3). These isolates were collected from multiple sources, including other food samples (3 %), but predominantly from humans (63 %). However, many of the publicly available genomes did not have information available regarding year of collection (38 %), source (29 %) and country of origin (67 %), leaving only 123 (16 %) genomes from human sources and the United Kingdom. These became the focus for determining if the *Y. enterocolitica* collected from food could be clinically significant.

3.5. Y. enterocolitica biotyping and serotyping

Phylogenetic analysis was able to predict the biotypes of 903/954 (95 %) of genomes (Fig. A.4). However, genomes belonging to biotypes 2 and 3 were found within the same clade, so isolates belonging to these clades were classified as biotype 2/3. Of the 207 food-derived isolates recovered as part of this study, 205 (99 %) were biotype 1A, and two were in a clade whose biotype was unknown (Fig. 3).

In silico serotyping predicted the serotype of 456/954 (48 %) of genomes. The serotypes were associated with biotypes: O:1,2,3 was only found in biotype 5, O:5,27 was only found in biotype 2/3 O:3 was only in biotype 4 or unknown biotypes, and O:13,7, O:5 and O:8 were only found in biotype 1A. Within biotypes, distantly related clades belonged to the same serotype. Of the 207 isolates recovered as part of this study, 7 (3 %) were O:13,7, 36 (17 %) were O:5, 61 (30 %) were O:8, and 103 (50 %) had an unknown serotype.

3.6. Genetic elements of interest

Analysis of AMR determinants in *Y. enterocolitica* genomes determined that 99 % of those from food contained the *blaA* gene that encodes a class A beta-lactamase conferring resistance to penam antimicrobials (Bent and Young, 2010), 2.4 % contained the *qacK* gene that encodes an efflux pump associated with resistance to quaternary ammonium compounds (QACs) and 99 % contained the *vat(F)* gene that encodes a streptogramin A acetyl transferase antimicrobial that confers resistance to streptogramin antimicrobials, compared to 94 %, 1 % and 96 % of the publicly available *Y. enterocolitica* from other sources investigated.

In terms of plasmid replicons, 3.3 % of those from food contained the Col(Ye4449).1 plasmid replicon, 0.5 % contained Col(YF27601).1, 4.8 % contained Col440II.1, 4.8 % contained ColE10.1, 0.5 % contained IncN.1, 0.5 % contained IncP6.1 and 13 % contained pYE854.1, compared to 0.5 %, 0 %, 0 %, 0.4 %, 2.1 %, 0 % and 4.0 % of the other *Y. enterocolitica* genomes investigated. However, none of the isolates collected from food contained the pYV plasmid (IncFII (Y).1_ps_CP001049 and IncFII(pYVa12790).1_pYVa12790_AY150843 reference sequences in PlasmidFinder), compared to 43.6 % of the other *Y. enterocolitica* genomes investigated.

Analysis of virulence genes amongst the *Y. enterocolitica* isolated from food and publicly available *Y. enterocolitica* identified 275 virulence genes, of which 123 were found in isolates recovered from food. Clustering of *Y. enterocolitica* genomes based on virulence genes identified two clusters consisting of isolates belonging to biotype 1B; two clusters consisting of isolates belonging to biotypes 2/3, 4 and 5; and multiple clusters consisting of isolates belonging to biotype 1A (Fig. A.5). Isolates from food and humans were distributed throughout the biotype 1A clusters (Fig. 4), but there were some distinctions between different serotypes within the biotype 1A clusters. Serotype distinctions were not evident amongst the non-biotype 1A clusters. Due to the large numbers of virulence genes associated with *Y. enterocolitica*, we focused on five virulence genes outlined by Hunter et al. (2019): *ail*, *inv*, *yadA*, *ystA* and *ystB* that are associated with biotypes (Table 1).

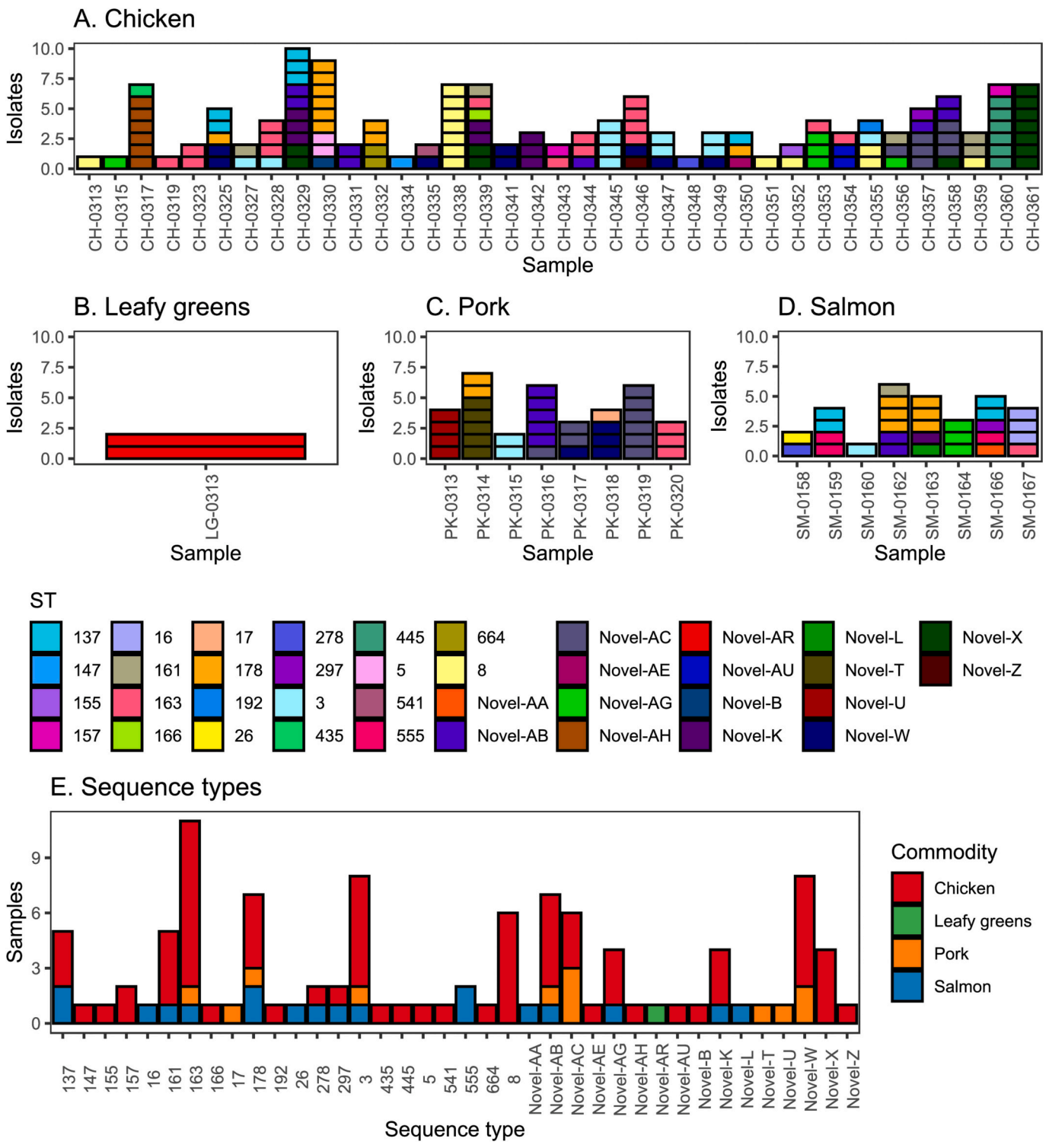

Fig. 2. Number of *Y. enterocolitica* isolates collected from positive chicken (A), leafy greens (B), pork (C) and salmon (D) samples and colored by ST. Number of samples that that were positive for each ST and colored by food commodity (E).

3.7. Within sample diversity

The 207 *Y. enterocolitica* isolates collected from food belonged to 38 STs, of which five STs were represented by a single isolate. Of the 54 food samples that were positive for *Y. enterocolitica*, 34 samples carried multiple STs: two STs were isolated from 21 samples, three STs were isolated from 10 samples, four STs were isolated from two samples, and five STs were isolated from one sample. For 40 food samples, multiple isolates belonging to the same ST were cultured, and isolates belonging to the same sample and ST differed by 0–74 core non-recombinant SNPs, 0–1 AMR determinants, 0–2 plasmid replicons and 0–10 virulence genes (Fig. A.6). Isolates belonging to different serotypes were from 26 food samples (Fig. A.7).

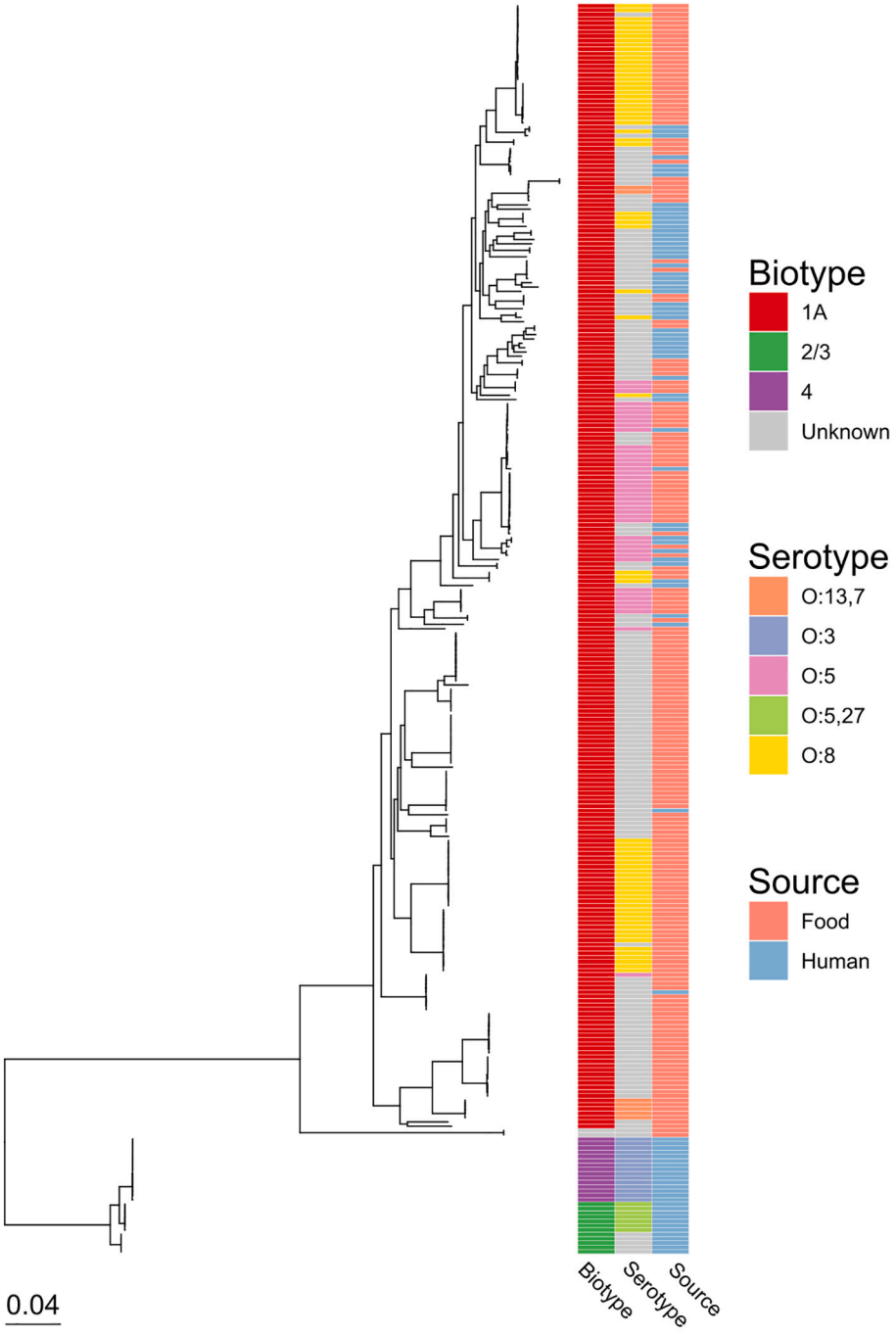

Fig. 3. Maximum-likelihood tree of *Y. enterocolitica* isolates collected from food and from UK human samples colored by biotype, serotype and source. The phylogenetic branch lengths are given in nucleotide substitutions per site, therefore a branch of length 0.04 (as represented by the scale bar) equates to 59,628 substitutions, given that the core gene alignment consisted of 1,490,689 bp.

3.8. Food-human Y. enterocolitica comparison

Of the 80 food samples tested for *Y. enterocolitica*, 41 samples contained isolates that belonged to the same ST that had previously been isolated from UK human sources (Fig. A.8). The smallest number of core non-recombinant SNPs between the food- and human-derived UK isolates for these samples ranged from 14 to 7978. The closest human UK isolates to isolates from food samples belonged to ST-8, ST-17, ST-555 and three novel STs (AC, AG and Z). The closest human-derived isolates were collected between 1993 and 2021 or the date of collection information was missing. For each of the serotypes isolated from food, O:5, O:8 and unknown serotypes were also identified from human UK sources, but no isolates from UK human-derived sources were available for O:13,7.

4. Discussion

Y. enterocolitica is found on food within a complex microbial community, therefore many selective methods have been developed to improve detection of this bacterium (Deboer, 1992). In this study we used methods based on the ISO standard (direct culturing, ITC broth and PSB broth) to culture for these bacteria. TPW broth was also initially used but was later removed to save time. Many STs were only isolated using one of these methods, but most of these method-specific STs were

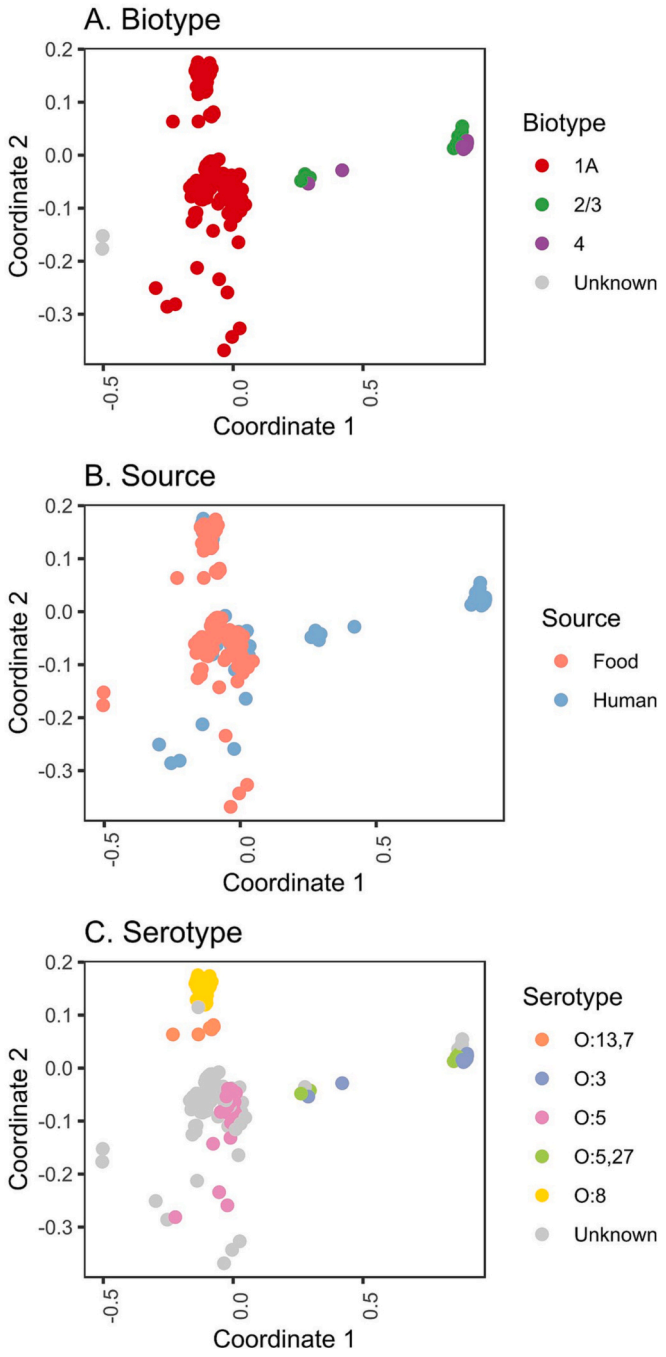

Fig. 4. Non-metric multidimensional scaling of *Y. enterocolitica* isolates collected from food and from UK human samples based on the presence of virulence genes and colored by biotype (A), source (B) and serotype (C).

only isolated from one sample. Without further sampling and comparisons with the other bacteria present on food, it is difficult to ascertain whether certain methodologies are better suited at isolating specific STs of *Y. enterocolitica* or if the results are due to variation in the methods' ability to inhibit other bacteria. Cheyne et al. (2009) applied similar culture methods to river water samples and found the methods were limited by their ability to inhibit other bacteria and that the best approach relied on using multiple methods to compensate for these limitations. Although we identified a diverse population of *Y. enterocolitica* in our samples by using multiple methods, we would likely have found an even more diverse population by incorporating further methods and possibly have identified *Y. enterocolitica* on more samples (Tan et al., 2014a).

Y. enterocolitica has traditionally been associated with pork (Martins et al., 2022). We isolated *Y. enterocolitica* from a large percentage of pork products (80 %), but also found similar proportions of *Y. enterocolitica* positive samples from chicken (74 %) and salmon (80 %). *Y. enterocolitica* from leafy greens was also identified in this study, but at a much lower proportion (10 %) than the meat and seafood commodities. However, an outbreak of *Y. enterocolitica* associated with leafy greens was reported in Norway (MacDonald et al., 2012), and unlike the other food commodities, leafy greens are unlikely to be cooked or processed further, so consumers are more likely to be exposed to the *Y. enterocolitica* present. Davies et al. (2001) found a similar proportion of salmon from Europe that contained *Y. enterocolitica* (80 %) using the methods recommended by the ISO standard as used in this study. Other *Y. enterocolitica* studies on food that did not utilize as many culture methods as our study identified lower proportions of positive samples from similar food commodities. Esnault et al. (2013) only used an ITC broth method and found *Y. enterocolitica* on 6.9 % of retail chicken and 5.2 % of pork samples from France. Ye et al. (2016) only used a PSB broth method and only found *Y. enterocolitica* on 14 % of chicken, 8.9 % of pork, 0.2 % of aquatic products and none of the fresh vegetables samples from China. Tan et al. (2014b) only used an ITC method and only isolated *Y. enterocolitica* from 12 % of the porcine products and none of the non-porcine products from Malaysia. In this study, we found larger proportions of retail food samples containing *Y. enterocolitica* compared to most previously published papers, but further global comparisons require standardized methodologies.

Y. enterocolitica has traditionally been typed based on biochemical tests into biotypes (Hunter et al., 2019). In this study we predicted *Y. enterocolitica* biotypes based on if they were in a clade of a genome with a known biotype. This predicted the biotype of 903/954 *Y. enterocolitica* genomes investigated, but led to problems with some clades that consisted of isolates belonging to both biotypes 2 and 3. Previous phylogenetic analysis of *Y. enterocolitica* belonging to these biotypes also identified them within the same clades (Reuter et al., 2014), supporting the argument that *Y. enterocolitica* should be described by their phylogroup and the virulence genes they contain (McNally et al., 2016). However, as many microbiologists use biotypes to describe *Y. enterocolitica*, we used these classifiers to compare *Y. enterocolitica* isolated from food.

Of the 207 *Y. enterocolitica* isolates obtained from food, 205 belonged to biotype 1A and two belonged to a clade of unknown biotype but closely related to biotype 1A. Biotype 1A is often considered non-

Table 1
Percentage of *Y. enterocolitica* containing virulence genes for different biotypes from this food study and other studies.

	Food		Other					
Biovar	1A	Unknown	1A	1B	2/3	4	5	Unknown
ail	0 %	0 %	0.6 %	89 %	99 %	100 %	100 %	69 %
inv	0 %	0 %	0 %	93 %	98 %	99 %	0 %	94 %
yadA	0 %	0 %	0 %	30 %	33 %	46 %	0 %	49 %
ystA	0 %	0 %	0 %	90 %	99 %	100 %	100 %	63 %
ystB	100 %	0 %	100 %	0 %	0 %	0 %	0 %	2 %
Number of genomes	205	2	312	27	121	231	7	49

pathogenic due to its lack of virulence genes. However, outbreaks associated with biotype 1A have been described (MacDonald et al., 2012). The high resolution offered by whole genome sequencing provides an opportunity to evaluate the potential pathogenicity of biotype 1A through comparison with isolates derived from clinical cases. In this study we identified *Y. enterocolitica* isolates from food that belonged to the same sequence type as those collected from UK human sources. However, not every *Y. enterocolitica* isolate collected from humans in the UK is genome sequenced, made publicly available and therefore included in this study. Effective source attribution of *Y. enterocolitica* will require improved detection and reporting of *Y. enterocolitica* from humans, and surveillance of food with sufficient isolates taken to capture the diverse populations present; this will also help determine the clinical significance of biotype 1A. In addition, just because highly similar *Y. enterocolitica* biotype 1A isolates were found in clinical and food specimens does not mean they were the agent responsible or that the isolate originates from that food type. Epidemiological evidence is required to determine likely sources of clinical infections (Greenwood and Hooper, 1990).

In addition to biotype, we predicted *Y. enterocolitica* serotypes *in silico* and found evidence of serotypes within biotype 1A containing similar virulence profiles despite being phylogenetically distinct. Outbreaks due to two of the serotypes isolated from food, O:5 (Ratnam et al., 1982) and O:8 (Shayegani et al., 1983), have been recorded, but we found no records of outbreaks due to O:13,7 (Tennant et al., 2003). Publicly available O:5 and O:8 genomes from human UK sources were closely related to those collected from food, but no O:13,7 genomes from human UK sources were available, suggesting O:13,7 may not be as clinically significant as the other serotypes identified in food in this study. As more *Y. enterocolitica* isolates are whole genome sequenced, *in silico* serotyping may help identify more clinically significant biotype 1A serotypes.

Understanding the diversity of *Y. enterocolitica* found on food samples is important for outbreak investigations as it informs how many isolates need to be sampled to capture the strain variation. Multiple studies have attempted to measure *Y. enterocolitica* diversity on food and animal sources (Shanmugapriya et al., 2014; Thong et al., 2018; Ye et al., 2015), but usually rely on PCR and PFGE-based methodologies that can discriminate at the biotype and serotype level. In this study we used WGS to go a step further and discriminate between isolates belonging to the same ST. The *Y. enterocolitica* isolates recovered from the same food samples in this study varied in diversity in terms of the number of STs of *Y. enterocolitica* isolated (1–5 STs) and the number of SNPs (0–74), AMR determinants (0–1), plasmid replicons (0–2), and virulence genes (1−10) shared amongst isolates recovered from the same sample and ST. This suggests that these food samples were contaminated with a diverse population of *Y. enterocolitica* at a single time point or at multiple time points with different strains. The large amount of diversity found amongst samples has implications for outbreak analysis, as sampling a single *Y. enterocolitica* isolate from a food sample will unlikely represent the diverse population present and could result in potential sources of infection being missed. It also explains how an outbreak with food as the vehicle could involve different strains of *Y. enterocolitica* (Inns et al., 2018). A similar finding was found with betel leaves imported into England that contained a diverse population of *Salmonella* belonging to different serovars, resulting in individual cases within outbreaks looking sporadic due to no common serovars (McLauchlin et al., 2019).

AMR is a growing problem and food has previously been found to contain a diverse population of AMR determinants (Bloomfield et al., 2023b). This study allowed us to determine if *Y. enterocolitica* from retail food contributes much to the potential reservoir of AMR genes on food. The chromosomal *blaA* and *vat(F)* genes were found in 99 % of *Y. enterocolitica* isolates from food, whilst the *qacK* gene was found in 2.4 % of isolates. This gene is associated with resistance to quaternary ammonium compounds (Huang et al., 2015), which are often used as a disinfectant in the food processing industry (Gerba, 2015), and their usage may be selecting for resistant bacteria in food. This suggests that outside of the core AMR determinants, the *Y. enterocolitica* we isolated do not contribute much to the AMR gene reservoir on food.

The presence of specific virulence genes can help indicate the pathogenicity of *Y. enterocolitica* strains. When looking at all virulence genes, biotype 1A *Y. enterocolitica*, to which most food isolates belonged, contained fewer virulence genes than did the other biotypes and clustered separately to these other biotypes based on virulence genes. However, we were unable to identify any group of biotype 1A isolates specifically associated with human isolates using virulence genes. All isolates we collected from food that belonged to biotype 1A contained *ystB*, whilst those that were of unknown biotype did not contain this gene. None of the isolates collected from food contained *ail, invA*, *yadA* or *ystA*, similar to the other publicly available 517 biotype 1A genomes we analyzed that all contained *ystB* but only two contained *invA*. Using PCR, Garzetti et al. (2014) found that the presence of the *ystB* and lack of the other virulence genes is associated with non-pathogenic strains. However, using WGS Hunter et al. (2019) identified highly pathogenic strains that lacked these genes. This suggests that the *Y. enterocolitica* pangenome is made up of large number of virulence genes, but further genomic work is required to determine which virulence genes are associated with more pathogenic biotype 1A strains.

This study found that 68 % of retail food samples tested were contaminated with *Y. enterocolitica*, much higher than previous reports investigating the presence of *Campylobacter* and *Salmonella* on retail foods (Bloomfield et al., 2023a; Korsak et al., 2015). However, *Y. enterocolitica* causes fewer reported infections than these bacteria (EFSA and ECDC, 2022). This study also found that food was contaminated with a diverse population of *Y. enterocolitica*, but most belonged to biotype 1A. This suggests that *Y. enterocolitica* biotype 1A is not as pathogenic as *Campylobacter* or *Salmonella*, yet biotype 1A has been isolated from many clinical samples (Clarke et al., 2020). In depth analysis of patients with biotype 1A could help determine if this biotype is causing infections and if there are any patient-specific predispositions to biotype 1A infections. This, in turn, could help identify biotype 1A strains associated with infections and genetic factors that allow them to cause infection.

5. Conclusion

In this study we investigated the genomic diversity of *Y. enterocolitica* on retail food. We identified diverse populations of *Y. enterocolitica* on some food types in terms of the number of STs present and number of SNPs shared amongst isolates belonging to the same ST. The best approach for culturing this bacterium relied on using multiple culture methods and could possibly contribute to our findings of higher proportions of *Y. enterocolitica* positive food samples than in most other studies that utilized a single culture method. Almost all the isolates collected from food belonged to biotype 1A, that has been described as non-pathogenic, but many of these isolates were closely related to those collected from human samples. Improved surveillance strategies of *Y. enterocolitica* from food and human sources is required to determine the role of biotype 1A in human disease.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110554.

CRediT authorship contribution statement

Raphaëlle Palau: Conceptualization, Investigation, Methodology, Writing – review & editing. **Samuel J. Bloomfield:** Conceptualization, Formal analysis, Methodology, Writing – original draft. **Claire Jenkins:** Writing – review & editing. **David R. Greig:** Software, Writing – review & editing. **Frieda Jorgensen:** Methodology, Writing – review & editing. **Alison E. Mather:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Genome data obtained as part of this study have been made publicly available on SRA

Acknowledgements

We gratefully acknowledge the Quadram Institute Bioscience core facilities sequencing and bioinformatics teams, laboratory technicians, media preparation and laboratory managers for their assistance in the project, and the CLIMB-BIG-DATA-computing servers, an infrastructure supported by the UK Medical Research Council grant MR/T030062/1.

Funding

This project was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Microbes in the Food Chain BB/R012504/1 and its constituent project BBS/E/F/000PR10348 (Theme 1, Epidemiology and Evolution of Pathogens in the Food Chain) and Food Standards Agency (FSA) project FS101185 through an FSA Fellowship to AEM. Further support was provided by BBSRC Institute Strategic Programme Microbes and Food Safety BB/X011011/1 and its constituent project BBS/E/F/000PR13634 (Theme 1, Microbial threats from foods in established and evolving food systems). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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