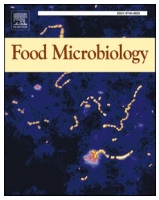
Food Microbiology 133 (2026) 104899

Contents lists available at ScienceDirect

Food Microbiology

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



Genomics uncover resistant and virulent *Klebsiella* on foods: a potential risk to human health

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ARTICLE INFO

Keywords:
Klebsiella
Antimicrobial resistance
Virulence
Hypervirulence
Food safety
Public health

ABSTRACT

Klebsiella pneumoniae is a bacterium of public health importance due to its association with antimicrobial resistance (AMR) and its role as a major cause of both hospital- and community-acquired infections. While *Klebsiella* species have been detected in foods, our understanding of their diversity and the potential risks they pose from food is limited. This study aims to comprehensively evaluate the *Klebsiella* species population and their contribution to the burden of AMR, virulence, and heavy metal tolerance from diverse food samples. We generated short-read sequence data for 570 *Klebsiella* isolates recovered from 361 food samples, including leafy greens, pork, prawns, chicken, salmon, and shellfish. Genome analysis showed that eleven unique *Klebsiella* species were present across food commodities, with *K. pneumoniae* being the most common (28.3 %); food-derived genomes were intermingled with publicly available *Klebsiella* genomes isolated from human clinical infections. We detected critical AMR genes, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{SHV-70}}$, and $bla_{\text{DHA-1}}$, in *K. pneumoniae* (n = 8) and *K. quasipneumoniae* (n = 6) from prawns. Additionally, we identified 46 virulent *K. pneumoniae* and *K. quasipneumoniae* isolates from domestic and imported food, including two hypervirulent *K. pneumoniae* isolates from domestic pork samples. Notably, a *K. planticola* isolate from salmon exhibited the hypermucoviscosity phenotype. AMR and virulence genes on plasmid contigs were widespread across different *Klebsiella* species, while chromosome-linked genes were mostly species-specific. These results highlight that food can harbour a range of *Klebsiella* species with resistance and virulence genes typically found in clinical settings, underscoring the need for monitoring foodborne *Klebsiella* as a potential risk to human health.

1. Introduction

Safe and nutritious food is essential to our health and well-being. However, unless specifically sterilised or processed, foods are covered in microbes, which may include pathogens and/or opportunistic pathogens (Janecko et al., 2023), which represents a potentially significant threat to public health (Mather et al., 2024). *Klebsiella* spp. are Gram-negative members of the Enterobacteriaceae family, ubiquitously found in a wide range of human and animal hosts and environmental niches across the One Health spectrum (Dong et al., 2022; Thorpe et al., 2022). *Klebsiella* spp. are normally commensal, but they have also been found to be opportunistic pathogens in hospital settings, responsible for infections of the urinary tract, soft-tissue, bloodstream, lower respiratory tract and more (Davis and Price, 2016; Wyres and Holt, 2016). Outside of hospital environments, *Klebsiella pneumoniae* in particular is a pathogen of note, causing severe community-acquired infections (Wyres et al., 2020). Aside from causing serious infections, *Klebsiella* species are known to acquire and disseminate antimicrobial resistance (AMR) and/or virulence traits, making them a significant public health concern due to the emergence of multidrug-resistant (MDR) and/or hypervirulent clones (Dong et al., 2022; Navon-Venezia et al., 2017).

K. pneumoniae is a dominant nosocomial opportunistic pathogen and has been widely studied due to the challenge in treating infections caused by MDR strains found in clinical settings, the invasive community-acquired infections caused by hypervirulent strains, and the

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https://doi.org/10.1016/j.fm.2025.104899
Received 24 April 2025; Received in revised form 7 August 2025; Accepted 8 August 2025
Available online 12 August 2025
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convergence of MDR-hypervirulent strains detected worldwide (Dong et al., 2022; Holt et al., 2015; Wyres and Holt, 2016). More recently due to an increased predominance of hypervirulent strains, further differentiation to supervirulent and ultravirulent phenotypes has been proposed (Douradinha, 2023; Tang et al., 2023). MDR *K. pneumoniae* is a serious threat as it has been seen to disseminate quickly across hospital settings leaving it hard to treat due to the acquisition of multiple AMR genes (Holt et al., 2015). The ability of *Klebsiella* spp. to acquire resistance to all classes of antimicrobials has been concerning, particularly as high rates of extended-spectrum cephalosporin-resistant and carbapenem-resistant *K. pneumoniae* have been detected globally (Navon-Venezia et al., 2017; Wyres et al., 2020; C. Yang et al., 2022). In addition, hypervirulent *K. pneumoniae* causes serious infections across multiple body sites in healthy immunocompetent individuals, clinically presenting as liver abscess, pneumonia, necrotizing fasciitis, meningitis and bacteraemia (Russo and Marr, 2019; Wyres et al., 2020). Hypervirulent strains are commonly characterized by a hypermucoviscous phenotype (observed as a positive "string test", which is the generation of a viscous string >5 mm in length from an isolated colony on agar plates), the carriage of virulence genes, such as yersiniabactin (*ybt*), aerobactin (*iuc*) and/or salmochelin (*iro*), genes associated with hypermucoidy (*rmpA*, regulator of mucoid phenotype A) and the predominance of KL1 and KL2 capsular types (Dong et al., 2022; Russo and Marr, 2019; Wu et al., 2017).

While the characteristics of MDR and hypervirulence have been more widely studied in *K. pneumoniae*, evidence of these traits have been also observed in other *Klebsiella* species. The carriage of MDR and multiple virulence factors have been reported in *K. variicola* (Farzana et al., 2019; Lu et al., 2018; Rodríguez-Medina et al., 2019), *K. quasipneumoniae* (C. Yang et al., 2022), species of the *K. oxytoca* species complex (SC) (Long et al., 2022; Moradigaravand et al., 2017; J. Yang et al., 2022), *K. ornitholytica* (Zou et al., 2020) and *K. planticola* (Li et al., 2022) from clinical samples, with the potential of these traits to be found in other *Klebsiella* species through mutation and/or horizontal gene transfer (J. Yang et al., 2022). Horizontal gene transfer events are often mediated by mobile genetic elements such as plasmids, integrons, transposons and insertion sequences (Partridge et al., 2018). Plasmids are extrachromosomal DNA elements that can encode genes and traits beneficial for their hosts and facilitate horizontal gene transfer between species and genera, which make their presence carrying AMR, virulence and/or heavy metal tolerance genes a significant public health concern (Navon-Venezia et al., 2017).

Although *Klebsiella* species have been most often studied in clinical or hospital settings, it is important to note that *Klebsiella* species harbouring resistance and virulence genes have also been identified in various animal hosts, including wildlife and livestock (Calland et al., 2023; Chiaverini et al., 2022; Mourão et al., 2023; Quintelas et al., 2024; C. Yang et al., 2022) as well as aquatic environments, such as wastewater, hospital effluents, marine settings, and seafood and fish (Håkonsholm et al., 2022; Li et al., 2024; Ludden et al., 2020; Thorpe et al., 2022; Vu et al., 2018; Zou et al., 2023). Despite not being recognized as a foodborne pathogen, *Klebsiella* spp. have also been detected on foods, such as retail meats, milk, fresh vegetables and ready-to-eat foods, as well as in food production facilities (Crippa et al., 2023; Davis and Price, 2016; Hartantyo et al., 2020; Klaper et al., 2021; Rodrigues et al., 2022; Wareth and Neubauer, 2021). In a retail food study done in the UK, the prevalence of *Klebsiella* spp. ranged between 3.2 % and 33.2 % in raw food commodities: leafy greens (33.1 %), chicken (21.9 %), pork (32.5 %), salmon (7.6 %) and prawns (26.5 %) (Janecko et al., 2023). However, there is still a limited understanding of the role of foods as a potential source for the transmission of AMR, virulent and hypervirulent *Klebsiella* species to humans.

Using WGS data from 570 *Klebsiella* spp. isolates from ready-to-eat (leafy greens), raw meat (chicken and pork), raw fish (salmon), raw prawn and shellfish commodities, this study aims to assess the risk of *Klebsiella* on foods to human health by: i) identifying signatures of *Klebsiella* species populations in food commodities, ii) characterizing the intra-sample *Klebsiella* species variation, and iii) assessing different *Klebsiella* species population and putative plasmids as a source of AMR, virulence, hypervirulence and heavy metal tolerance.

2. Materials and methods

2.1. Study design for the Klebsiella isolate collection

A previously reported repeated cross-sectional study was conducted between May 2018 and November 2019 in the county of Norfolk in the United Kingdom (UK), where key food commodities – chicken, pork, leafy greens, prawns and salmon – were collected to assess the prevalence of *Klebsiella* spp. and other bacteria (Janecko et al., 2023). The microbiological detection and isolation of *Klebsiella* spp. from these food commodities was described previously, with up to two isolates collected per sample (Janecko et al., 2023). This generated 564 *Klebsiella* spp. isolates from 357 food samples. In addition to this dataset, six isolates from four shellfish samples were included, which were sampled by the Food Water and Environmental Microbiology Services, National Infection Service, UK Health Security Agency (UKHSA) in Norfolk in 2019. Subsequently, the overall *Klebsiella* spp. collection for this study comprised 570 isolates from 361 samples, of which 152 (42.1 %) samples had a single isolate recovered per sample and 209 (57.9 %) samples had two isolates recovered per sample.

2.2. Whole genome sequencing and Klebsiella species identification

DNA extraction for short-read sequencing was performed using the Maxwell RSC Cultured Cells DNA kit (Promega, Southampton, UK). Preparation of paired-end libraries were made using the Nextera XT DNA library kit and libraries were sequenced as 150bp paired-end reads on an Illumina NextSeq (Illumina, Inc., San Diego, CA, USA). Trimmomatic v0.33 (Bolger et al., 2014) was used on the raw short-reads to remove adapters and poor-quality bases, and the trimmed reads were used to assemble draft genomes with Spades v3.11.1 (Bankevich et al., 2012) using the –careful option, which performs a mismatch correction. Summary statistics of the assemblies were obtained using Quast v4.6.3 (Gurevich et al., 2013), such as number of contigs, total length and %GC content. CheckM v1.1.3 (Parks et al., 2015) was used to assess the genome quality through the completeness and contamination information. BWA v0.7.17 (Li and Durbin, 2009), Samtools v1.9 (Li et al., 2009) and Bcftools v1.8 (Danecek et al., 2021) were used to map reads against their corresponding assemblies for the largest four contigs to estimate mean chromosomal sequencing depth coverage.

The *Klebsiella* species assignation was performed using Kleborate v2.3.2 (Lam et al., 2021). In addition, FastANI v1.32 (Jain et al., 2018) was used to estimate the average nucleotide identity (ANI) of all orthologous genes shared between pairs of genomes, and with this approach the species and subspecies assignations were confirmed. The sequence type (ST) was obtained for each genome with ARIBA v2.14.6 (Hunt et al., 2017) using the *K. pneumoniae* multilocus sequence type (MLST) scheme for *K. pneumoniae* SC, *K. oxytoca* MLST scheme for *K. oxytoca* SC and *K. aerogenes* MLST scheme for only *K. aerogenes*, which were available from pubMLST (Jolley and Maiden, 2010).

2.3. Screening of AMR, virulence and metal tolerance genes in chromosome and plasmid contigs

AMR genes, virulence genes and plasmid replicons were identified in short-read data through ARIBA v2.14.6 (Hunt et al., 2017) using the Resfinder (Bortolaia et al., 2020), vfdb_core (Chen et al., 2016) and Plasmidfinder (Carattoli et al., 2014) databases, respectively. To identify the exact location of the AMR and virulence genes in the contigs, the same Resfinder (Bortolaia et al., 2020) and vfdb_core (Chen et al., 2016) databases and blastn option of Blast + v2.9.0 (Camacho et al., 2009)

were used. Genes conferring tolerance to metals and/or antibacterial biocides were screened in the draft genomes using the BacMet2 (Pal et al., 2014) database and the tblastn option of Blast + v2.9.0 (Camacho et al., 2009). The threshold to detect virulence and metal tolerance genes using blastn was 95 % identity and 95 % coverage; for the AMR genes, a 90 % identity threshold was used to match the settings used with ARIBA v2.14.6 (Hunt et al., 2017). Furthermore, aerobactin variant, capsule K-locus (KL) and O-antigen types were identified through Kleborate v2.3.2 (Lam et al., 2021).

RFPlasmid v0.018 (van der Graaf-van Bloois et al., 2021) was used on the draft genomes to predict the contig as chromosome or plasmid origin; a default cut-off of ≥0.5 was used to classify the contigs. The plasmid subtyping was performed using Blast + v2.9.0 (Camacho et al., 2009) and the plasmid MLST (pMLST) database (https://bitbucket.org/genomicepidemiology/pmlst_db/) for the IncF plasmid types. Due to the multi-replicon (FII, FIA and FIB replicons) nature of the IncF plasmids, a replicon sequence typing scheme (RST) was used through the FAB formulae (FII:FIA:FIB) (Villa et al., 2010).

2.4. Screening of key virulence factors and string test for the hypermucoviscosity phenotype

To identity hypervirulent *Klebsiella* variants phenotypically, three indicators were used: a string test positive result, detection of *rmpA* and detection of aerobactin genes. Isolates with at least two of these three indicators were considered as hypervirulent, as previously described (Wu et al., 2017). To identify potential virulent *Klebsiella*, ten key virulence factors were screened in the whole genome collection: mucoviscosity-associated gene A (*mag/wzy_K1*), K-locus 1 (KL1), mucoid phenotype A regulator (*rmpA*), aerobactin (*iucABCD*), salmochelin (*iroBCDN*), yersiniabactin receptor (*irp*1-2), yersiniabactin synthesis (*ybtAEPQSTUX*), enterobactin (*entABCDEF*), fimbriae (*mrkABCDHIJF*) and allantoinase (*allABCDRS* and *fyuA*). Combinations of these virulence factors were defined as virulence factor profiles.

A set of isolates (n = 53) was selected on which to perform the string test, the criteria of selection being: 1) all isolates (n = 47) carrying key virulence factor profiles with ≥3 different virulence factors, and 2) a subset of isolates (n = 6) without key virulence factor profiles (Table S6) as a comparator. This latter subset was limited to six isolates since none harboured key virulence factors, making them less likely to be hypervirulent variants even if they were hypermucoviscous. Using the string test previously described by Wu et al. (2017), the 53 *Klebsiella* isolates were assessed for the hypermucoviscosity phenotype. Briefly, the isolates were subcultured on Columbia blood agar with 5 % sheep's blood (Trafalgar Scientific, Leicester, UK) at 37 °C for 24 h ± 3 h. The *Klebsiella* isolates were considered as hypermucoviscous if, when stretching the colonies using an inoculation loop, the viscous string was >5 mm in length.

2.5. Analysis of genomic relatedness within foodborne Klebsiella species and with human-derived Klebsiella species

The draft genomes were annotated with Prokka v1.13.3 (Seemann, 2014) and the pangenome was assessed with Roary v3.12.0 (Page et al., 2015), where core genes were identified with a threshold of 90 % identity in 95 % of the isolates. snp-sites v2.5.1 (Page et al., 2016) was used to extract the single nucleotide polymorphisms (SNPs) from the core gene alignment and the invariant sites information, this latter obtained with the -C parameter. These SNP data and invariant sites were used in IQ-TREE v1.6.11 (Nguyen et al., 2015) to build a maximum likelihood tree with ModelFinder Plus (MFP) option to determine the best substitution model for the data, which resulted in the general time reversible (GTR) plus FreeRate (+R10) model. Branch support was evaluated through 1000 ultrafast bootstraps (UFBoot) (Hoang et al., 2018) implemented in IQ-TREE V1.6.11 (Minh et al., 2020). The phylogenetic tree was constructed for the whole *Klebsiella* genome collection (n = 570).

To understand the genomic relatedness of *Klebsiella* species isolated from food in this study in a public health context, the BV-BRC database (Olson et al., 2023) was queried for human-derived genomes of the 11 *Klebsiella* species. Kleborate v2.3.2 (Lam et al., 2021) was used to confirm species and determine *K. pneumoniae* species complex STs. Only complete and WGS entries were considered and to reduce the number of *K. pneumoniae* genomes, one genome was randomly selected to represent STs with more than three genomes in the retrieved dataset, and a random selection was done to reduce the numbers of other species. Only *K. grimontii, K. michiganensis, K. ornithinolytica, K. pasteurii,* and *K. planticola* human-derived genomes were not subsampled. Mashtree v1.4.6 (Katz et al., 2019) was then used to create the tree which was plotted with the ggtree v3.10.1 (Yu et al., 2017) R package.

3. Results

3.1. Genome collection of Klebsiella species from food

Genome data were obtained for a collection of 570 *Klebsiella* spp. isolated from 361 food samples, including leafy greens, pork, prawns, chicken, salmon and shellfish. Eleven recognized *Klebsiella* species were identified, with *K. pneumoniae* (n = 161; 28.3 % of total isolates) the most common, followed by *K. planticola* (n = 98; 17.3 %), *K. ornithinolytica* (n = 94; 16.5 %), *K. michiganensis* (n = 61; 10.7 %), *K. grimontii* (n = 38; 6.7 %), *K. quasipneumoniae* subsp. *similipneumoniae* (n = 33; 5.8 %), *K. variicola* subsp. *variicola* (n = 23; 4 %), *K. oxytoca* (n = 21; 3.7 %), *K. pasteurii* (n = 20; 3.5 %), *K. quasipneumoniae* subsp. *quasipneumoniae* (n = 17; 3 %) and *K. aerogenes* (n = 2; 0.4 %). To note, in this study the *Raoultella* species are referred to as *Klebsiella* species as they share an average nucleotide identity (ANI) of 84.1 (range: 83.5–85.5) and this is above the cut off for genus boundaries observed in *Escherichia* (ANI of 81.42) and *Enterobacter* (ANI of 83.17) (Barco et al., 2020), which is consistent with previous studies in *Klebsiella* (Ma et al., 2021; Thorpe et al., 2022; Wyres et al., 2020). In addition, two genomes from one sample (leafy greens) were labelled as *Klebsiella* Ko11 by Kleborate v2.3.2 (Lam et al., 2021); Ko11 has been proposed as a new *Klebsiella* species (Ma et al., 2021). Given the low numbers and being a new taxon, these two Ko11 genomes were removed from further analysis and only were included in the phylogenetic tree analysis to show their relatedness to the *K. oxytoca* SC. The draft genomes for all 570 *Klebsiella* isolates from 361 food product samples passed quality control (Supplementary Dataset S1, Fig. S1 and Fig. S2).

Of the 360 food samples examined, 152 (42.2 %) samples yielded one *Klebsiella* isolate per sample and 208 (57.8 %) samples yielded two isolates per sample. To assess any potential bias of the latter set of samples on the relative proportion of the *Klebsiella* species recovered, the frequency of each species recovered was calculated in: 1) a 'singleton' subset comprising the 152 samples with a single isolate per sample (n = 152 isolates), 2) a 'doubleton' subset comprising the 208 samples with two isolates per sample (n = 416 isolates), and 3) the entire collection (n = 568 isolates without the two Ko11 isolates). Across all three datasets, *K. pneumoniae* was consistently the most frequently recovered species, and its proportion, along with those of the other *Klebsiella* species, remained comparable (Fig. 1A–B, Table S1 and Fig. S3). This consistency indicates that samples containing multiple isolates per sample ('doubleton' subset) did not alter the *Klebsiella* species proportion compared to the 'singleton' subset. In addition, within the 'doubleton' dataset, different *Klebsiella* species were found in only 23.1 % (48/208) of samples, whereas 76.9 % (160/208) of samples contained *Klebsiella* of the same species (Fig. 1C).

3.2. Distribution of Klebsiella species across food commodities

Klebsiella species were found in diverse food commodities (Fig. 1A–B); *K. pneumoniae*, *K. michiganensis*, *K. pasteurii*,

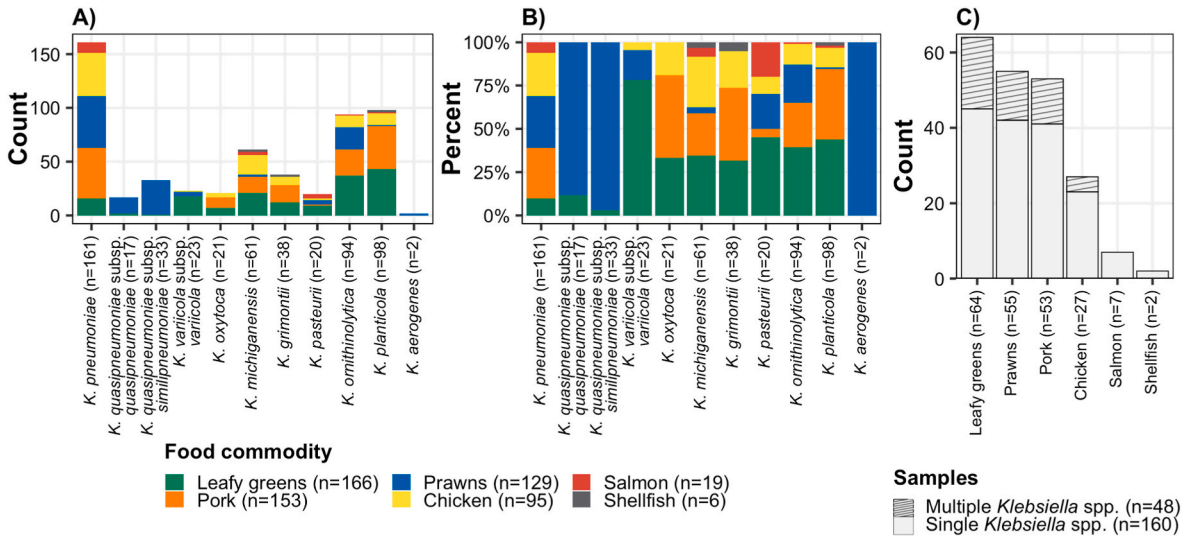

Fig. 1. Distribution of *Klebsiella* species across food commodities. **A)** Count and **B)** percentage of food commodities with each *Klebsiella* species (n = 568 isolates) (colored as per inset legend). **C)** Set of food samples (n = 208) with two isolates per sample: number of samples with single *Klebsiella* species per sample (grey without diagonal lines), and number of samples with multiple *Klebsiella* species per sample (grey with diagonal lines).

K. ornithinolytica and *K. planticola* were detected in five tested food commodities – leafy greens, pork, prawns, chicken and salmon; furthermore, the three latter *Klebsiella* species were also found in shellfish (Fig. 1A–B and Table S2). The other *Klebsiella* species were all found in at least three different food commodities. An association was observed between some food commodities and *Klebsiella* species, such as: *K. variicola* subsp. *variicola* was predominantly found in leafy greens, while *K. quasipneumoniae* subsp. *quasipneumoniae* and subsp. *similipneumoniae* were mainly found in prawns (Fig. 1A–B and Table S2). These patterns were consistent in the singleton and doubleton datasets (Fig. S3).

3.3. Population structure of Klebsiella species and intra-sample variation

The phylogenomic evolution of the *Klebsiella* species were assessed using SNPs from the core gene alignment, which was built using 2111 core genes found in 95 % of the genomes. The core gene alignment (2,120,581 bp) represented 37.3 % of the average genome length (5,680,334 bp) across *Klebsiella* species. Through this SNP-based phylogenetic tree the four *Klebsiella* SC, as previously described (Thorpe et al., 2022), were identified: 1) *K. pneumoniae* SC including *K. pneumoniae*, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, and *K. variicola* subsp. *variicola*; 2) *K. aerogenes* SC including only *K. aerogenes*; 3) *K. oxytoca* SC including *K. oxytoca*, *K. michiganensis*, *K. grimontii*, *K. pasteurii* and *K.* Ko11 (the two Ko11 genomes were removed from further analysis); and 4) *K. ornithinolytica* SC including *K. ornithinolytica* and *K. planticola* (Fig. S4). The genome population structure of the *Klebsiella* species (Fig. 2A) agreed with the species assignation by Kleborate v2.0.4 (Lam et al., 2021); genomes belonging to the same species had ANI >97 % (Fig. S5), genomes within the same SC had ANI values between 91.2 and 96.9 % and between-SC genomes had ANI between 83.5 and 86.4 % (Fig. S6).

Of the 208 samples with two isolates per sample, most samples (n = 160) had isolates of the same *Klebsiella* species; these included isolates that belonged to the same ST as well as to different STs (Fig. 2A). Conversely, for the samples that exhibited co-occurrence of two different *Klebsiella* species (n = 48), the most common combination was *K. ornithinolytica* and *K. planticola* (n = 12 samples from leafy greens, pork and chicken) (Fig. 2B). Additionally, different species complexes were also observed within individual samples; for instance, *K. pneumoniae* and *K. ornithinolytica* (n = 3 samples from pork).

3.4. Resistome, virulome, metal tolerance and plasmid types

A total of 128 different AMR genes were found, potentially conferring resistance to 14 distinct antimicrobial classes. The majority of these (75 genes) were located in chromosomal contigs, conferring resistance to nine antimicrobial classes, while 45 genes were predicted to be carried on plasmids, conferring resistance to 13 antimicrobial classes; eight genes were found in both chromosome and plasmid contigs (Fig. S7 and S8). MDR (carrying genes conferring resistance to ≥3 different antimicrobial classes) was mostly observed in *K. pneumoniae* SC isolates (95.3 %; 223/234), and rarely observed in other *Klebsiella* SC, such as *K. oxytoca* SC isolates (7.1 %; 10/140) and *K. ornithinolytica* SC isolates (7.3 %; 14/192) (Fig. 3A). Almost all isolates (566 out of 568) from the *Klebsiella* species, except *K. aerogenes*, carried at least one AMR gene of chromosomal origin, and additionally, 90 out of 568 isolates from different *Klebsiella* species carried at least one AMR gene of plasmid origin. An association between certain AMR genes and *Klebsiella* species was observed, with particular chromosomally encoded AMR genes specific to a *Klebsiella* species (i.e., bla_{LEN} in *K. variicola* subsp. *variicola*, bla_{OKP} in *K. quasipneumoniae*, bla_{SHV} in *K. pneumoniae*, bla_{OXY} in *K. oxytoca* SC and bla_{PLA} in *K. ornithinolytica* SC); in contrast, plasmid-encoded AMR genes were detected in multiple *Klebsiella* species (Fig. S9). Critically important AMR genes were identified, such as the extended-spectrum beta lactamase (ESBL) $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$, $bla_{SHV\text{-}70}$ and AmpC $bla_{DHA\text{-}1}$ genes conferring resistance to extended-spectrum cephalosporin antibiotics (Kochan et al., 2022); $bla_{CTX\text{-}M\text{-}15}$ was found in plasmid contigs from *K. pneumoniae* and *K. quasipneumoniae* subsp. *quasipneumoniae* isolates from prawns (Fig. 3D and Table S3), and $bla_{CTX\text{-}M\text{-}27}$ was detected in two *K. quasipneumoniae* subsp. *similipneumoniae* isolates from two prawn food samples, but in a chromosome contig (Table S3). $bla_{SHV\text{-}70}$ was found in chromosome contigs from *K. pneumoniae* derived from prawns, whereas $bla_{DHA\text{-}1}$ was located in plasmid contigs from both *K. pneumoniae* and *K. quasipneumoniae* subsp. *quasipneumoniae*, also isolated from prawns. The *mcr-9* gene was found in *K. pneumoniae*, *K. ornithinolytica*, *K. oxytoca* and *K. michiganensis* isolates from chicken, leafy green and shellfish samples.

In *Klebsiella*, the majority of virulence factors are features encoded by accessory genes, and their presence can increase the severity of infections and/or propensity to cause disease (Wyres et al., 2020). In this study, 103 genes encoding virulence were identified and were classified into 14 different virulence factors. *K. pneumoniae* SC carried more

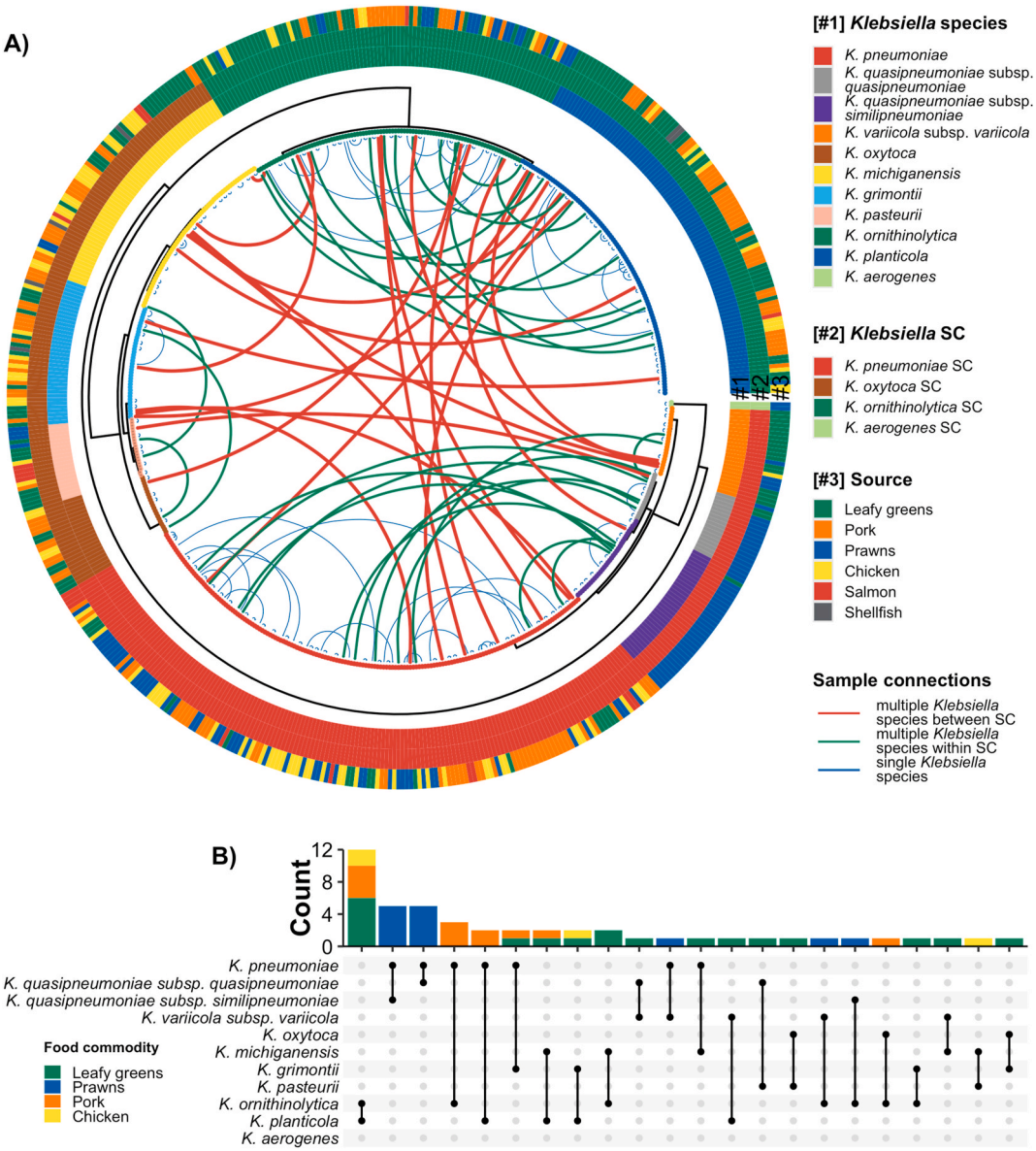

Fig. 2. Maximum-likelihood core gene tree of 568 *Klebsiella* isolates from food commodities and intra-sample variation. **A)** Phylogenetic tree: in the center of plot, arcs connect genomes isolated from the same sample: blue arcs connect single *Klebsiella* species per sample, green arcs connect multiple *Klebsiella* species within SC per sample, and red arcs links multiple *Klebsiella* species between SC per sample. Ring from inner to outer: *Klebsiella* species, *Klebsiella* SC (species complex) and food commodity. **B)** Distribution of samples with multiple species per sample across food commodities (colored as per inset legend). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

virulence factors compared to the other *Klebsiella* SC (Fig. 3B). The majority of virulence factors were located in the chromosome, with those that were found in plasmid contigs identified in multiple *Klebsiella* species (Fig. S10); specifically, type 3 fimbriae (encoded by *mrkABCDFHIJ*) was found across different *Klebsiella* SC, while aerobactin (*iucABCD*), yersiniabactin receptor (*irp*1-2), and yersiniabactin synthesis (*ybtAEPQSTUX*) were only identified in *K. pneumoniae* (Fig. 3E). To note, all 10 aerobactin-positive isolates had the *iuc3* variant, with nine isolates recovered from pork samples, and one from a chicken sample. With respect to metal and/or antibacterial biocide tolerance, 65 genes were identified encoding tolerance primarily to arsenic, copper, nickel, mercury, silver and tellurium; 69.9 % (397/568) of the isolates carried genes conferring tolerance to at least one of these metals. Silver and copper tolerance genes were commonly found in plasmid contigs from 250 isolates across multiple *Klebsiella* species (Fig. S11) and different food commodities (Fig. S12).

A total of 45 different plasmid replicons were detected among the isolates, with 73.9 % (420/568) carrying at least one plasmid replicon. The identified plasmid replicons primarily belonged to nine plasmid Incompatibility (Inc) types, of which IncF (46.0 %, n = 388) was the most frequent. Less frequently identified were IncR, IncHI1, IncN, IncX, IncC, IncHI2, IncY and IncI1 (Fig. 3C–Table S4). For the dominant IncF plasmid type, a subtyping analysis was performed, classifying these as K17:A-:B-, K5:A-:B-, K2:A-:B-, K12:A-:B-, Y7:A-:B-, K3:A-:B-, Y6:A-:B-, K9:A-:B-, K1:A-:B- and K4:A-:B-, which represented 42.5 % of the IncF plasmid replicons. Rare putative subtypes represented 14.5 % of the IncF plasmid replicon occurrences (Fig. 3F–Table S5).

3.5. Virulent, hypervirulent and hypermucoviscosity Klebsiella variants

There were seven combinations, or profiles, of key virulence factors, some of which comprised a single virulence factor and others up to eight virulence factors (Fig. S13 and Table S6). Five profiles contained ≥3 different virulence factors (referred here as key virulence factor profiles)

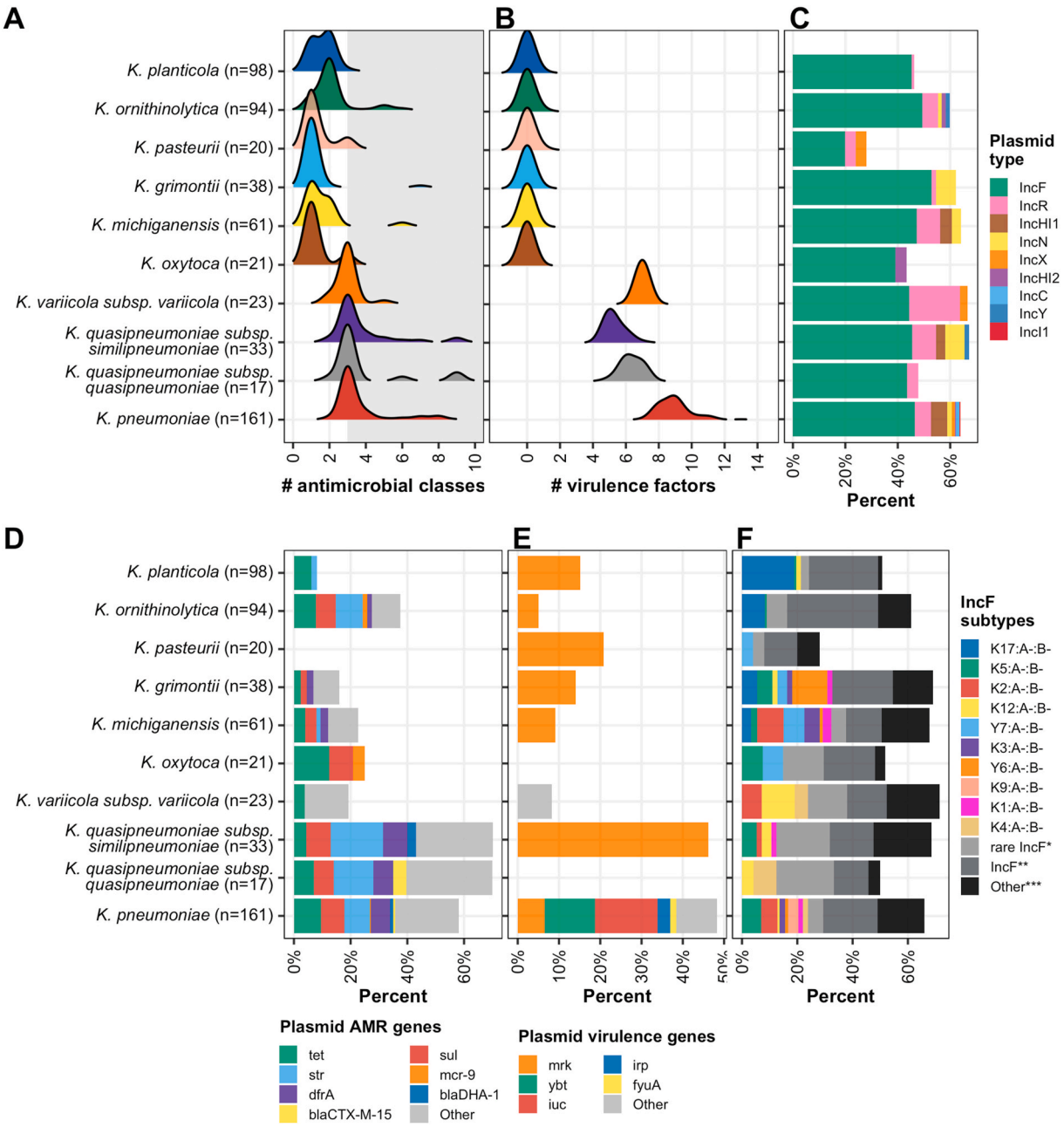

Fig. 3. Resistance, virulence and plasmid types/subtypes for 566 *Klebsiella* isolates. Distribution of the **A)** number of antimicrobial classes, **B)** virulence factors, and **C)** proportion of plasmid Inc types by *Klebsiella* species. Proportion of **D)** antimicrobial resistance (AMR) genes and **E)** virulence genes of plasmid origin. **F)** Proportion of IncF plasmid subtypes. To note: the two isolates of *K. aerogenes* were not included due to the low sample number. Details of the genes shown in panel D and E are as follows: *tet* (*tet*(A-D, G)), *sul* (*sul1-3*), *str* (*strA-B*), *dfrA* (*dfrA1*, *12*, *14–17*, *23*, *27*), *mrk* (*mrkABCDFJ*), *ybt* (*ybtAEPQSTUX*), *iuc* (*iucABCD*) corresponding to *iuc3* variant, *irp* (*irp1-2*). Panel A and B are colored by *Klebsiella* species, and panel C–F are colored as per inset legend.

and represented 8.3 % (47/568) of the whole collection and were found in *K. pneumoniae*, *K. quasipneumoniae* subsp. *quasipneumoniae* and *subsp. similipneumoniae* (Fig. S13A) from diverse food commodities (Fig. S13B). 32.9 % (187/568) of the collection carried two different virulence factors, 1.8 % (10/568) carried one virulence factor and 57.0 % (324/568) did not contain any key virulence factors (Table S6). In this study, isolates that exhibited ≥3 different virulence factors were classified as virulent *Klebsiella* variants.

A hypermucoviscosity phenotype was assessed in all *Klebsiella* isolates (n = 47) carrying ≥3 different target virulence factors, but also in isolates (n = 6) without the key virulence factors (Table S6). Only one of these *Klebsiella* isolates was hypermucoviscous but did not contain any target virulent factors, a *K. planticola* from salmon (Fig. S14).

Two *K. pneumoniae* ST692 isolates from a UK-origin pork sample carried the *rmpA* (regulator of mucoid phenotype A) and aerobactin (*iucABCD*) genes – specifically the *iuc3* variant - which are indicators of a hypervirulent variant (Wu et al., 2017). These two hypervirulent isolates also carried six additional key virulence factors (salmochelin [*iroBCDEN*], yersiniabactin receptor [*irp*1-2], yersiniabactin synthesis [*ybtAEPQSTUX*], enterobactin [*entABCDEF*], type 3 fimbriae [*mrkABCDFHIJ*] and allantoinase [*fyuA*]) (Fig. S13). However, while genetically matching the hypervirulent genotype, these two isolates were phenotypically negative by the string test. With respect to AMR, these two isolates carried only chromosomal AMR genes, including bla_{SHV-99}, *fosA* and *oqxAB*. Another important virulence factor, *magA/wzy_K1* (mucoviscosity-associated gene A), was found in only two

K. quasipneumoniae subsp. *quasipneumoniae* ST196-3LV isolates from an imported prawn sample; these isolates also carried other key virulence factors such as enterobactin (*entBEF*) and type 3 fimbriae (*mrkABC*) and were capsule serotype KL1 (Fig. S13). These two isolates were also negative by the string test (string <5 mm in length).

3.6. Public health context of foodborne Klebsiella

The genomic relatedness between the 568 foodborne *Klebsiella* genomes, belonging to 11 *Klebsiella* species, and 2138 *Klebsiella* genomes derived from human hosts, which were retrieved from the BV-BRC database, was examined. For the human-derived genomes with known isolation sources, the majority (1743/1757) were clinically related (i.e., blood, urine, wound, abscesses and others), while for 381 genomes, the isolation source was unknown (Supplementary Dataset S2). Each *Klebsiella* species formed a monophyletic cluster, with the food-derived genomes distributed within the clusters with those of human origin, indicating close relatedness. However, some human-specific clusters were also observed (Fig. 4 and Fig. S15). There were fewer human-derived *K. planticola* and *K. ornithinolytica* genomes than food-derived genomes compared to all the other *Klebsiella* species.

4. Discussion

In this study, diverse *Klebsiella* population species were identified from key commonly consumed food commodities, with the important human pathogens *K. pneumoniae* and the *K. pneumoniae* SC the most frequently detected. Although it is difficult to compare with other studies on food as many of these focus solely on *K. pneumoniae* (Hartantyo et al., 2020; Rodrigues et al., 2022), other *Klebsiella* species have been previously found in different food commodities, such as *K. quasipneumoniae* in cattle, milk and fish, *K. variicola* in cattle, milk, poultry and fresh vegetables, *K. oxytoca* in retail meats, poultry and fresh vegetables, *K. michiganensis* and *K. grimontii* in cattle, milk and poultry, *K. ornithinolytica* in cheese and salami and retail meats and *K. planticola* in cheese and salami (Biggel et al., 2021; Crippa et al., 2023; Falomir et al., 2013; Gundogan et al., 2011; Klaper et al., 2021; Messaoudi et al., 2009; Nüesch-Inderbinen et al., 2023; Sajeev et al., 2022). These individual findings, along with the results of this study, demonstrate the diverse *Klebsiella* species that can be encountered in various food commodities. Although *Klebsiella* is known to be ubiquitous, this study observed specific patterns in the distribution of certain species; *K. variicola* subsp. *variicola* was mostly identified in leafy greens, as seen previously (Holt et al., 2015), whereas *K. quasipneumoniae* subsp. *quasipneumoniae* and subsp. *similipneumoniae* were commonly found in prawns. In a dairy farm setting, *K. pneumoniae* was reportedly linked with rumen and faecal content, while *K. oxytoca*, *K. variicola* and *K. planticola* were linked with soil and feed crops (Zadoks et al., 2011). The co-occurrence of two different *Klebsiella* species within a single food sample in this study suggests possible contamination of *Klebsiella* species along various points in the food chain or the contamination of a food sample with multiple species, with further potential for transmission and infection in humans. The close genomic relatedness of the foodborne *Klebsiella* species and those clinically related in humans provides added

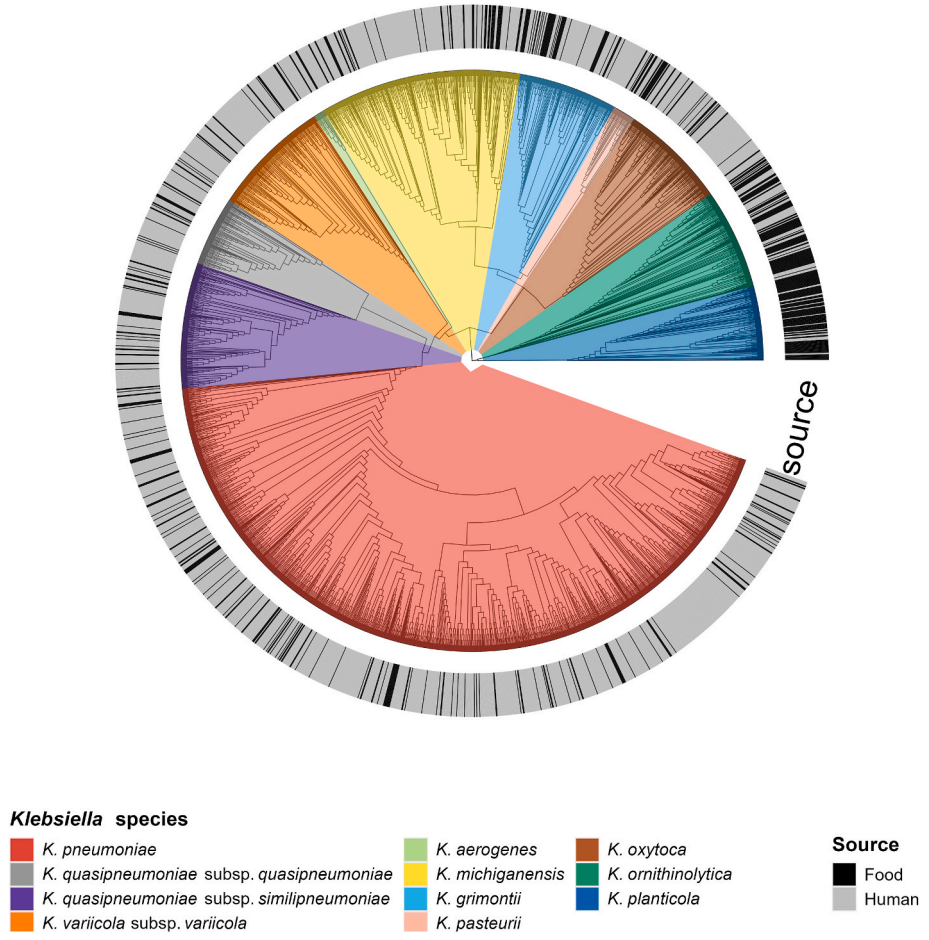

Fig. 4. Genomic relatedness among *Klebsiella* isolates (n = 2706) from human and food sources. Human-derived genomes (n = 2138) were retrieved from the BV-BRC database, and the food-derived genomes were from this study (n = 568). The branches of the tree are colored by *Klebsiella* species, and the outer ring shows genome source.

evidence that foodborne *Klebsiella* species potentially contribute to the public health burden.

Nearly all *Klebsiella* isolates (99.6 %; 566/568) in this study contained at least one predicted chromosomally encoded AMR gene, while 16.8 % (95/566) of isolates carried AMR genes on plasmid contigs. The majority of the AMR genes identified confer resistance to twelve different antimicrobial classes; aminoglycoside, beta-lactam, fluoroquinolone, fosfomycin, lincosamide, macrolide, phenicol, quinolone, rifampicin, sulphonamide, tetracycline and trimethoprim. *Klebsiella* species have intrinsic resistance to penicillin through the production of different beta-lactamases (Dong et al., 2022), genes for which were observed on the chromosome in this study, including bla_{SHV} in *K. pneumoniae*, bla_{OKP} in *K. quasipneumoniae*, bla_{LEN} in *K. variicola*, bla_{OXY} in *K. oxytoca* SC and bla_{PLA} in *K. ornithinolytica* SC (Holt et al., 2015; Klaper et al., 2021; Walckenaer et al., 2004; Wyres et al., 2020; J. Yang et al., 2022). In addition to beta-lactamases, high numbers of *fosA* genes associated with fosfomycin resistance and *oqxAB* genes associated with quinolone resistance were detected on chromosome contigs across isolates of six and four different *Klebsiella* species in this study, respectively. Although the numbers are potentially concerning as both antibiotics are used in the treatment of various infections, the genetic context of these genes needs to be considered to understand their phenotypic resistance and ultimately their clinical impact. In *Klebsiella*, some variants of these fosfomycin genes are found in the chromosome and their expression under natural promoters do not confer phenotypic clinical resistance. In contrast, clinical resistance to fosfomycin is observed when the mobilization of the *fosA3* gene is located on a plasmid and is regulated by a constitutive promoter derived from IS*26* elements (Kieffer and Guzmán-Puche, 2024). In this study, all *fos* variants were found in the chromosome, which is unlikely to lead to a fosfomycin resistant phenotype but could result in a reduced susceptibility as reported by Wyres et al. (2020). Similarly, the chromosomal-borne *oqxAB* confers a reduced susceptibility to quinolones, but the mobile forms of these genes can confer clinically relevant resistance (Wyres et al., 2020). This highlights the importance of AMR genes' genetic context and location (chromosome/plasmid) in understanding phenotypic clinically relevant resistance.

A small number of isolates (3.3 %; 19/568) harboured critically important AMR genes such as ESBLs ($bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$ and $bla_{SHV\text{-}70}$) and AmpC $bla_{DHA\text{-}1}$, which were found in *K. pneumoniae* or *K. quasipneumoniae* isolates from prawns. These ESBL and AmpC genes, conferring resistance to the critically important extended-spectrum beta-lactam antibiotics, have been previously found in human clinical samples (Calland et al., 2023; Hawkey et al., 2022; Ling et al., 2006; Ludden et al., 2020; Thorpe et al., 2022), and ESBL-producing *Klebsiella* species have been previously detected in seafood (Håkonsholm et al., 2020; Vu et al., 2018). Additionally, the *mcr-9* gene, conferring potential resistance to colistin, was detected in three different *Klebsiella* species isolated from chicken, leafy greens and shellfish. Although initially thought to confer resistance to colistin, studies have shown that only a few isolates carrying *mcr-9* have phenotypically displayed colistin resistance at the EUCAST epidemiological cut-off value of 2 mg/L. More recent studies have potentially identified genes that could induce *mcr-9* expression, but further research would be needed to determine the clinical impact of *mcr-9* carrying *Klebsiella* species and therefore only genotypic potential colistin resistance was reported in this study (Carroll et al., 2019; Macesic et al., 2021; Tyson et al., 2020). The detection of *Klebsiella* species in foods carrying ESBLs, AmpC and *mcr* genes leading to extended-spectrum cephalosporin and colistin resistance, respectively, are of concern as these have public health implications; these resistant *Klebsiella* species could cause infections in humans that are difficult to treat and additionally result in the further spread of resistance. With the increasing use of carbapenems and colistin as last resort treatments for infection by MDR bacteria, these results highlight potential One Health concerns that need to be monitored and further studied. Regarding MDR *Klebsiella*, we found it mainly in *K. pneumoniae* SC isolates and less frequently in other *Klebsiella* species. This has also been reported worldwide, with rising occurrences of MDR across other *Klebsiella* species complexes, though mainly in clinical settings and not in food (Farzana et al., 2019; Holt et al., 2015; Moradigaravand et al., 2017; Rodríguez-Medina et al., 2019).

Virulent and hypervirulent *Klebsiella* species were found in this study, with species of the *K. pneumoniae* SC harbouring more virulence genes compared to the rest of the *Klebsiella* species detected, which has also been previously observed by Holt et al. (2015). Similar to other studies, aerobactin genes (*iucABCD*), which in this study were all the *iuc3* variant, were observed in *K. pneumoniae* on pork and poultry samples, while yersiniabactin genes (*irp1-2* and *ybtAEPQSTUX*) were found in *K. pneumoniae* isolated from a range of food commodities (Crippa et al., 2023; Franklin-Alming et al., 2021; Klaper et al., 2021; Thorpe et al., 2022). This result supports previous findings suggesting pork is a potential source of *Klebsiella* carriage and transmission to humans due to the presence of the aerobactin virulence factor (Crippa et al., 2023; Franklin-Alming et al., 2021; Klaper et al., 2021; Thorpe et al., 2022). Foods can be potential vehicles for the transmission of virulent *Klebsiella* species to humans; this was seen by Davis et al. (2015), where similar virulence profiles were isolated from *Klebsiella pneumoniae* strains from retail meats and clinical specimens, which could be the same in the results in this study. Only two hypervirulent (carrying *rmpA* and *iucABCD*, the latter corresponding to the *iuc3* variant) *K. pneumoniae* isolates were detected in this study from a domestic pork sample, which also carried several additional virulence factors and chromosome-borne AMR genes. Hypervirulent *Klebsiella* strains are community-associated, with reports showing that they are becoming increasingly resistant through the acquisition of mobile elements carrying AMR genes (Russo and Marr, 2019). While hypervirulent strains with AMR genes on mobile elements were not detected in this study, they have been isolated from retail meats and seafood previously, adding further evidence for food to be a reservoir of virulent and hypervirulent *Klebsiella* species with the potential for transmission to humans (Håkonsholm et al., 2022; Sabala et al., 2024). Although only the type 1 and 3 fimbriae were detected in this study, other fimbrial gene clusters have been reported in *Klebsiella* species. These include the *Escherichia coli* common pilus (ECP), the *Salmonella enterica* Serovar Typhi fimbrial proteins *sth,* π fimbrie and the *kpf* gene cluster, which could support persistence through biofilm formation and increased virulence (Di Mento et al., 2022; Gomes et al., 2021; Villa et al., 2017). In this study, from a subset of isolates that were tested using the 'string test', only a single *K. planticola* isolate that had no identified virulence factor genes displayed the hypermucoviscous phenotype, while two isolates carrying genes associated with mucoviscosity, *mag/wzy_K1*, produced negative string tests. This supports the more current consensus that hypermucoviscosity does not equate to hypervirulence, and that these are two distinct phenotypic characteristics (Catalán-Nájera et al., 2017; Dong et al., 2022). Hypermucoviscosity is characterized by capsule production, which has been found to be associated with *K. pneumoniae* fitness, and the hypermucoviscous phenotype and overproduction of the capsular polysaccharide has been shown to potentially provide a fitness advantage for invasive infections (Mike et al., 2021). Our study is the first to report a *K. planticola* isolate with the hypermucoviscous phenotype, which has mainly been seen in *K. pneumoniae* and within the *K. pneumoniae* SC, in *K. variicola* (Catalán-Nájera et al., 2017) and *K. quasipneumoniae* subsp. *similipneumoniae* (Garza-Ramos et al., 2016). This is of clinical importance as *K. planticola*, though normally found in water and soil environments, has been increasingly reported to cause infections particularly in immunocompromised and paediatric patients (19).

Metal and/or antibacterial biocides can co-select for AMR and therefore the presence of genes that confer tolerance to metal/biocides represent a selection pressure for AMR (Baker-Austin et al., 2006). These metal/biocide and AMR genes can co-occur on the same mobile genetic element, causing dissemination and the potential spread of AMR in

various niches (Anedda et al., 2023). Heavy metals naturally occur in the environment but also are used in animal feed or in aquaculture against biofouling, and resistance genes can be introduced to the environment through faecal contamination (Koutsoumanis et al., 2021). Mourão et al. (2023) reported the co-occurrence of copper and colistin resistance genes on *K. pneumoniae* plasmids, similar to those found in human clinical isolates, along the poultry production chain. This finding suggests foods are a possible reservoir of *Klebsiella* species carrying plasmids with genes with clinical implications. In this study, genes encoding tolerance to heavy metals were detected in 69 % of all isolates across *Klebsiella* species and food commodities, with silver and copper tolerance genes found in plasmid contigs from 250 *Klebsiella* isolates, indicating the potential for either the acquisition or dissemination of AMR and/or metal tolerance genes through possible co-selection.

Klebsiella species are notorious for their role in the acquisition and dissemination of AMR and/or virulence genes to other bacteria in the same environment, which are mediated by the horizontal spread of plasmids carrying these genes (Navon-Venezia et al., 2017). In this study, around 74 % of isolates had at least one plasmid replicon, and IncF plasmids were the most common across all species. IncF plasmids are commonly found in species of Enterobacteriaceae and frequently carry resistance and virulence genes (Holt et al., 2015; Navon-Venezia et al., 2017; Stein et al., 2024). In this study, AMR genes, virulence factors and metal/biocide tolerance genes that were found on plasmid contigs were widespread across different *Klebsiella* species and food commodities, and some critical resistance and virulence genes, such as $bla_{\text{CTX-M-15}}$, $bla_{\text{DHA-1}}$ and aerobactin *iuc3* variant (*iucABCD)*, were found solely on plasmid contigs indicating the potential transfer of these genes to other bacteria on the same food sample (Navon-Venezia et al., 2017; Zhang et al., 2023). Previous studies have also detected ESBL-carrying plasmids in *Klebsiella* species in seafood, and plasmid-borne aerobactin genes in pigs and pork, suggesting that seafood or the marine environment and pigs/pork are reservoirs of plasmids carrying critical resistance and virulence genes, respectively (Crippa et al., 2023; Håkonsholm et al., 2020; Kaspersen et al., 2023; Klaper et al., 2021; Thorpe et al., 2022).

5. Conclusions

The isolation of *Klebsiella* species from food commodities, combined with WGS, has enabled us to reveal the diverse population of *Klebsiella* found in contaminated food samples. Our findings highlight four key aspects: 1) *K. pneumoniae* is the most frequently identified species on food, and multiple distinct *Klebsiella* species can coexist on a single food sample. 2) *K. pneumoniae* and *K. quasipneumoniae* isolates from prawns can carry critically important ESBL and AmpC genes, which have been previously reported across human clinical, environmental and food sources (Calland et al., 2023; Davis et al., 2015; Hawkey et al., 2022; Thorpe et al., 2022). 3) We identified virulent (with $\geq$3 different virulence factors) *K. pneumoniae* and *K. quasipneumoniae* in domestic and imported food commodities, and hypervirulent *K. pneumoniae* from a UK-origin pork sample. This implies that key resistance and virulence genes carried by *Klebsiella* species can be found across the One Health spectrum, with foods as potential reservoirs. This, together with the close genomic relatedness of food- and human-derived strains, warrants further research into foodborne *Klebsiella* and their potential clinical impact, particularly in the spread of resistance and virulence and their contribution to the public health burden. Additionally, our study is the first to report the hypermucoviscosity phenotype in *K. planticola*. Finally, 4) Plasmid-encoded AMR and virulence genes were detected in multiple *Klebsiella* species across various food commodities. This indicates that certain plasmids with clinically significant genes are highly mobile, although to note, with the short-read sequencing utilized here we were not able to obtain fully contiguated plasmid sequences, for which long-read sequencing is required (Hernandez et al., 2024; Zamudio et al., 2024). Further research on foodborne plasmids would help uncover the potential role of mobile genetic elements in the spread of AMR and virulence across different host species, as well as their potential contribution to clinical infections.

CRediT authorship contribution statement

Roxana Zamudio: Writing – original draft, Visualization, Formal analysis. **Min Yap:** Writing – original draft, Visualization, Formal analysis. **Samuel J. Bloomfield:** Writing – review & editing, Methodology, Investigation. **Raphaëlle Palau:** Writing – review & editing, Methodology, Investigation. **Nicol Janecko:** Writing – review & editing, Investigation. **Alison E. Mather:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Funding

This project was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) through the 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), Food Standards Agency (FSA) project FS101185 through an FSA Fellowship to AEM and BBSRC Institute Strategic Programme Microbes and Food Safety BB/X011011/1 and its constituent project BBS/E/QU/230002A (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.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alison Mather reports financial support and article publishing charges were provided by the Biotechnology and Biological Sciences Research Council. Alison Mather reports financial support was provided by the Food Standards Agency. Alison Mather reports a relationship with the Biotechnology and Biological Sciences Research Council that includes: consulting or advisory, funding grants, and travel reimbursement. Alison Mather reports a relationship with the Food Standards Agency that includes: consulting or advisory and funding grants. Alison Mather reports a relationship with the University of East Anglia that includes: employment. Nicol Janecko reports a relationship with the Food Standards Agency that includes: consulting or advisory and travel reimbursement. Alison Mather reports a relationship with the Medical Research Council that includes: funding grants. Alison Mather, Samuel Bloomfield have a patent pending to Quadram Institute Bioscience. All other 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.

Acknowledgments

We thank the Food Water and Environmental Microbiology Services at the UK Health Security Agency for providing the four shellfish samples. We gratefully acknowledge use of the Quadram Institute Bioscience (QIB) core facilities HPC cluster servers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.104899.

Data availability

All raw Illumina short-reads generated in this study have been deposited in the European Nucleotide Archive (ENA) with 513 genomes

under BioProject PRJEB62598 and 57 genomes under BioProject PRJNA1135353. Metadata and individual accession numbers are available in the Supplementary Dataset S1.

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