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Arsenic bioaccumulation in ready-to-eat oysters can contribute to the selection of WHO critical priority Enterobacterales displaying a virulent behavior

Felipe Vásquez-Ponce [a,b,*], Nicolas Gamboa-Acuña [c], Aline Pereira de Oliveira [d], Johana Becerra [a,b], Jesus G.M. Pariona [b,e], Leon de Oliveira Lima [f], Aline de Carvalho Elias [d,g], Maciel Santos Luz [g], Mateus Rocha Ribas [h], Gustavo Rocha [h], Fernanda Esposito [b,e], Patricio Muñoz [i], Edson Gonçalves Moreira [j], Cassiana Seimi Nomura [d], Thais Sincero [h], Edison Barbieri [k], Nilton Lincopan [a,b,e,*]

[a] Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[b] One Health Brazilian Resistance Project (OneBR), São Paulo, Brazil
[c] Departamento de Desarrollo, INSACOM SpA., Valparaíso, Chile
[d] Instituto de Química, Universidade de São Paulo, São Paulo, Brazil
[e] Department of Clinical Analysis, School of Pharmacy, University of São Paulo, São Paulo, Brazil
[f] Departamento de Química, Universidade Federal de São Paulo, Diadema, Brazil
[g] Laboratory of Metallurgical Process, Institute for Technological Research, São Paulo, SP, Brazil
[h] Laboratório de Microbiologia Molecular Aplicada, Departamento de Análises Clínicas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, Trindade, Florianópolis, Santa Catarina, Brazil
[i] Institute of Geociences, University of São Paulo, São Paulo, Brazil
[j] Nuclear and Energy Research Institute, IPEN/CNEN, São Paulo, Brazil
[k] Instituto de Pesca, Agência Paulista de Tecnologia dos Agronegócios (APTA), Secretaria de Agricultura e Abastecimento, de São Paulo, (SAASP) - Governo do Estado de São Paulo, Brazil

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ABSTRACT

The bioaccumulation of arsenic (As) in shellfish poses a serious threat to human health, whereas the occurrence of antimicrobial-resistant bacteria in seafood raises food safety concerns. We investigated the occurrence and genomic background of WHO critical-priority Enterobacterales in ready-to-eat (RTE) oysters, and their association with As bioaccumulation. RTE oysters collected between September 2022 and March 2023, in five Brazilian markets, were analyzed. In brief, oyster tissue samples were aseptically removed and processed for isolation and identification of carbapenem- and/or third-generation cephalosporin-resistant Enterobacterales, using MALDI-TOF (Bruker) and disk diffusion methods. Heavy metal tolerance was evaluated by microdilution assays. Quantification of As, Hg, Cu, Co, Ag and Pb was performed by inductively coupled plasma optical emission spectrometry and atomic absorption spectrometry. Enterobacterales were sequenced by Illumina NextSeq, and virulence was evaluated using the *Galleria mellonella* infection model. Statistical analysis was made with Phyton. Total As concentrations in RTE oysters ranged from 0.44 to 1.95 mg/kg. Five As-tolerant (MIC≥1024 μg/mL) and multidrug-resistant Enterobacterales producing extended-spectrum beta-lactamases (ESBLs) were identified. WGS confirmed the *ars* operon among international clones of *Klebsiella pneumoniae* (ST307) coproducing CTX-M-15 and SHV-28, *Escherichia coli* (ST38 and ST23) producing CTX-M-15 and CTX-M-55, and *K. quasipneumoniae* (ST526) producing SHV-5 ESBLs. *Citrobacter telavivensis* producing CTX-M-15 in seafood is reported for the first time. *E. coli* ST38 and ST23 exhibited highly virulent behavior and phylogenomic relationships with human lineages. The occurrence of WHO priority pathogens and As bioaccumulation in oysters is a public health issue that requires surveillance and appropriate management strategies.

* Corresponding authors at: Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil.
E-mail addresses: felipe.vasquez@icb.usp.br (F. Vásquez-Ponce), lincopan@usp.br (N. Lincopan).

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1. Introduction

Food safety remains a critical global challenge, exacerbated by rapid population growth and ongoing development. The demand for food is projected to increase by 35–56 % by 2050, raising concerns about the potential for widespread malnutrition if sustainable solutions are not implemented (Dillirani Nagarajan et al., 2024; Van Dijk et al., 2021).

Oysters (*Crassostrea gigas*) are the most consumed shellfish seafood globally (Martínez-García et al., 2022), particularly in their raw form (Botta et al., 2020). Oysters are highly nutritious, containing glycogen, protein, polyunsaturated fatty acids, and minerals (Zhu et al., 2018). However, consuming raw oysters poses inherent food safety risks due to their filter-feeding nature, which enables them to bioaccumulate a wide range of contaminants, including potentially toxic heavy metals, pathogenic microorganisms and antimicrobial-resistant bacteria (Guedes et al., 2023; Rao et al., 2024; Kijewska et al., 2023). In this regard, as filter feeders, oysters can process large volumes of water, potentially concentrating bacteria from contaminated aquatic environments. This is particularly worrying in estuarine and coastal waters impacted by human activities, where untreated or partially treated sewage, agricultural runoff, and industrial waste introduce antimicrobial residues and resistant bacteria (Pavón et al., 2022; Xiao et al., 2023). These environments often serve as reservoirs for multidrug-resistant (MDR) bacteria, posing significant risks to consumers who ingest raw or undercooked seafood (Milijasevic et al., 2024; Reverter et al., 2020).

Arsenic (As) is classified as one of the most toxic elements to human health, according to the 2024 Hazardous Substances Priorities List issued by the Agency for Toxic Substances and Disease Registry (ATSDR, 2024). Chronic exposure to ingested As is particularly concerning due to its strong association with carcinogenesis, primarily affecting the skin, bladder, and lungs (Hughes et al., 2011). Anthropogenic activities constantly lead to discharge of significant amounts of As along marine coastlines; therefore shellfish generally contain higher concentrations of As than other foods (Rahman et al., 2012). This harmful heavy metal could be transferred by oysters and other edible bivalves through the food chain. The bioaccumulation of As in oysters poses a potential dietary health risk to humans (Kato et al., 2020). Bioaccumulation is the process by which organisms, including oysters, accumulate contaminants at higher concentrations than those in their environment, influenced by uptake mechanisms like respiration and diet, as well as elimination processes such as metabolism and excretion. The bioaccumulation includes bioconcentration; where contaminants transfer directly from water to the organism, and biomagnification; which occurs through dietary intake, leading to increased contaminant levels at higher trophic positions in the food web (Borga, 2013).

Antimicrobial resistance (AMR) is an increasing challenge that extends beyond the medical and veterinary fields to also impact on the food industry, with possible risks to both food quality and safety (Wu-Wu et al., 2023). The 2021–2025 Action Plan on AMR by the Food and Agriculture Organization of the United Nations (FAO) outlines five key strategic priorities aimed at addressing this issue within the food and agriculture sectors: (i) raising awareness and fostering stakeholder engagement; (ii) advancing surveillance and research to support evidence-based policymaking; (iii) promoting practices to prevent infections and mitigate the spread of resistance; (iv) ensuring the prudent use of antimicrobials to preserve their efficacy; and (v) reinforcing governance with adequate resource allocation. Among these priorities, surveillance stands as the cornerstone for understanding antibiotic resistance dynamics in food, enabling the detection of emerging threats, and guiding targeted interventions to mitigate the spread of resistant microorganisms. (Keck et al., 2023). In this regard, clinically relevant MDR Enterobacterales producing extended-spectrum beta-lactamases (ESBL) and/or carbapenemases have been designated as critical-priority pathogens by the World Health Organization (WHO), since they pose a significant public health risk (Tacconelli et al., 2018).

Recently, the potential role of seafood, including oysters, as a source for disseminating critical-priority Enterobacterales has begun to be recognized as a significant challenge to public health (Mohammed et al., 2024; Vásquez-Ponce & Barbieri, 2025). In this regard, while some studies have documented the contamination of raw-cut fish, from retail markets, with pathogenic and antibiotic-resistant bacteria (Amin et al., 2024; Xedzro et al., 2025), as well as the transcontinental dissemination of Enterobacterales harboring carbapenemase genes in retail frozen shrimp (Parker et al., 2025), fatal seafood-borne carbapenem-resistant infections have begun to be reported (Sawasdichai et al., 2025), underscoring a serious risk, particularly in immunocompromised patients.

Since most microbiological studies in seafood have focused on foodborne pathogens, following current FAO recommendations, we investigated the occurrence and genomic background of WHO critical priority Enterobacterales in ready-to-eat (RTE) raw oysters, and their association with heavy-metal and As bioaccumulation.

2. Materials and methods

2.1. Sample collection and processing

Oysters (n = 108) belonging to the species *Crassostrea gigas* and *Crassostrea brasiliana* were collected from five markets (M1, M2, M3, M4 and M5) across São Paulo and Santa Catarina states, in Brazil, during the winter of 2022 (September) and the summer of 2023 (March) (Table 1). The oysters were immediately transported to the laboratory in ice boxes at a controlled temperature of 4 °C. Individuals of similar size from each site were selected for analysis. A total of 12 live, closed oysters were collected per market, rinsed with distilled water, and aseptically opened inside a sterile laminar flow cabinet. All sample processing was

Table 1
Antimicrobial-resistant Enterobacterales isolated from oysters collected from five markets during the winter and summer seasons of 2022 and 2023, in Brazil.

Market	State, City, Coordinates	Oyster species	Resistant bacterial strains isolated in winter 2022 [i]	Resistant bacterial strains isolated in summer 2023 [i]
M1	São Paulo, Cananéia (S25°01′09.3" W 47°55′33.3″)	*Crassostrea brasiliana*	*Escherichia coli* [d, f], *Aeromonas* spp. [e], *Proteus* spp. [h]	*Escherichia coli* [g], *Morganella morganii* [f, g], *Pseudomonas otitidis* [h], *Aeromonas* spp. [c], *Comammonas aquatica* [d]
M2	São Paulo, Santos (S23°59′04.3" W46°17′42.6″)	*Crassostrea brasiliana*	*Klebsiella pneumoniae* [a*]	*Escherichia coli* [a*, h*], *Aeromonas* spp. [b], *Proteus* spp. [c]
M3	São Paulo, São Paulo (S23°33′56.2" W46°41′33.5″)	*Crassostrea gigas*	NG	*Morganella morganii* [f], *Proteus* spp. [g]
M4	Santa Catarina, Florianópolis (S27°39′48.7" W48°30′08.6″)	*Crassostrea gigas*	*Klebsiella pneumoniae* [a*] *Citrobacter* spp.[a*]	NG
M5	São Paulo, Peruíbe (S24°19′48.9" W47°00′10.0″)	*Crassostrea brasiliana*	NS	*Escherichia coli* [d, e, g, h] *Pseudomonas aeruginosa* [a]

Bacterial growth in antibiotic selective media: a; ceftriaxone (2 μg/mL), b: ceftazidime/avibactam (2 μg/mL) c; meropenem (1 μg/mL), d; ciprofloxacin (1 μg/mL), e; gentamicin (1 μg/mL), f; colistin (1 μg/mL), g; oxytetracycline (4 μg/mL), h; florfenicol (4 μg/mL. *; ESBL phenotype by disc approximation test. NG: No bacterial resistant growth. NS: Not sampled. i; Bacterial identification was performed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

performed by the same individual to ensure consistency throughout the study. Approximately 100 g of tissues from each collection were pooled into sterile Whirl-pak® sampling bag (Nasco, Fort Atkinson, WI). The pooled soft tissue was then fragmented with a sterile scalpel and homogenized using a Stomacher Smasher™ AESAP1064 (bioMérieux, United States) for 2 min. Aliquots of 25 g were transferred to new sterile plastic bags and mixed with 225 mL of peptone water, creating a 1:10 dilution. The mixtures were incubated aerobically at 37 °C and 100 rpm for 4 h to enrich bacterial cultures. After this initial incubation, 1 mL from each sample was transferred into separate tubes containing 9 mL of peptone water, each supplemented with a single antibiotic at the following final concentrations: colistin (COL, 1 μg/mL), vancomycin (VAN, 1 μg/mL), oxacillin (OXA, 1 μg/mL), ceftazidime-avibactam (CZA, 2 μg/mL), ceftriaxone (CRO, 2 μg/mL), meropenem (MER, 1 μg/mL), gentamicin (GEN, 1 μg/mL), ciprofloxacin (CIP, 1 μg/mL), oxytetracycline (OXY, 4 μg/mL), and florfenicol (FLOR, 4 μg/mL), focusing mainly on the isolation of WHO critical-priority Enterobacterales (Bertagnolio et al., 2024; Tacconelli et al., 2018). These antibiotics were selected to represent distinct clinically relevant antimicrobial classes. Meropenem (carbapenems), ceftriaxone (third-generation cephalosporins), and ceftazidime/avibactam (β-lactam/β-lactamase inhibitors) were included to assess β-lactam resistance mechanisms. Gentamicin (aminoglycosides) and ciprofloxacin (fluoroquinolones) were selected as key agents against multidrug-resistant bacteria. Oxytetracycline (tetracyclines) and florfenicol (phenicols) are commonly used in veterinary medicine, particularly in aquaculture. Colistin (polymyxins) was included due to its role as a last-resort antibiotic for multidrug-resistant Gram-negative bacteria. Vancomycin (glycopeptides) and oxacillin (penicillins) were selected to target resistance mechanisms in Gram-positive bacteria. Each tube was incubated for an additional 24–48 h. After this incubation, 10 μL of each bacterial culture was plated onto MacConkey, TCBS, mannitol, and M-Enterococcus agar plates. The plates were incubated at 37 °C for 24–48 h, and each medium was supplemented with a disc of the corresponding antibiotic from the enrichment broth to assess the presence of resistant bacteria. Colonies growing near each antibiotic disc were selected for further analysis.

2.2. Bacterial identification, antimicrobial susceptibility, heavy metal tolerance and phenotypic detection of extended-spectrum beta-lactamases

Bacterial identification and antimicrobial susceptibility testing were performed using MALDI-TOF (Bruker) and disk diffusion methods, respectively. The antibiotics (μg/disk) tested included aztreonam (ATM, 30), ampicillin (AMP, 10), amoxicillin (AMO, 10), piperacillin (PIP, 30), ticarcillin (TIC, 75), amoxicillin/clavulanic acid (AMC, 20/10), piperacillin/tazobactam (PPT, 30/6), cefoxitin (CFO, 30), cefotaxime (CTX, 30), ceftazidime (CAZ, 30), ceftazidime/avibactam (CZA, 14 and 50), ceftriaxone (CRO, 30), cefepime (CPM, 30), imipenem (IMP, 10), ertapenem (ETP, 10), meropenem (MEM, 10), tetracycline (TET, 30), doxycycline (DO, 30), chloramphenicol (C, 30), florfenicol (FLOR, 30), ciprofloxacin (CIP, 5), nalidixic acid (NAL, 30), gentamicin (GEN, 10), streptomycin (SPT, 10), fosfomycin (FOS, 50), and sulfamethoxazole/trimethoprim (SUT, 25). Results were interpreted according to the Performance Standards for Antimicrobial Disk Susceptibility Tests (Clinical and Laboratory Standards (CLSI), 2023) and the European Committee on Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2023) guidelines. *Escherichia coli* ATCC 25922 was used as a control for the antimicrobial susceptibility tests. The ESBL production was evaluated for isolated strains by the disk approximation method (Dias et al., 2014). On the other hand, heavy metal tolerance was evaluated using a broth microdilution method (CLSI, 2023) for mercury chloride ($HgCl_2$; Mallinckrodt, UK), sodium arsenite ($NaAsO_2$; Baker & Adamson, USA), silver nitrate ($AgNO_3$; Merck, Germany), copper sulfate ($CuSO_4{\cdot}5H_2O$; Synth, Brazil), potassium dichromate ($K_2Cr_2O_7$; Vetec, Brazil) and cobalt chloride ($CoCl_2$; Baker & Adamson, USA). The heavy-metal-tolerant *Klebsiella pneumoniae* strain KPN535 (Gaeta et al., 2022), which harbors mercury (*merA-C*), arsenic (*arsA-D*, *arsH*), silver (*silR-S*, *silC*, *silE*), copper (*pcoA-E*, *pcoR-S*), and nickel (*nikA-E*) resistance genes, and *E. coli* ATCC 25922, which lacks genes conferring heavy metal tolerance, were used as control strains.

2.3. Heavy metal quantification in oyster tissue by inductively coupled plasma optical emission spectrometry and atomic absorption spectrometry

Approximately 5 g of tissue from 12 oysters was freeze-dried using a lyophilizer *E*-C Micro Module (Thermo, Germany) for 48 h and ground using a mortar and pestle. The ground tissue was stored in polypropylene tubes at −4 °C. Subsequently, acid digestion was performed on the ground samples using a microwave (Multiwave Go Plus model, Anton Paar GmbH, Graz, Austria) equipped with a twelve-position rotor (12HVT50, Anton Paar GmbH, Graz, Austria). Multi-element determination was performed by inductively coupled plasma optical emission spectrometry (ICP OES), using an iCAP 6300 Duo spectrometer (Thermo Fisher Scientific, Darmstadt, Germany), equipped with axial and radial plasma views. A charge-injection device detector was used, allowing measurements from 166.25 to 847.00 nm. An echelle polychromator was purged with argon with a purity of 99.998 % (v v^{-1}) (Oxilúmen, Brazil), and a radiofrequency source of 27.12 MHz was used. For arsenic (As), cadmium (Cd), chromium (Cr), and silver (Ag) determination using ICP OES, the samples underwent microwave-assisted acid digestion with 2 mL of 65 % nitric acid (HNO_3), 0.5 mL of 30 % hydrogen peroxide (H_2O_2), and 3 mL of deionized water. For the acid digestion, the following heating program was used: A 10-min ramp period was used to reach 190 °C, followed by a 5-min hold. After cooling for 10 min to room temperature, the vials were cooled to 40 °C before depressurization. Digestion was performed in triplicates for each sample and after digestion, each digested sample was diluted to a final volume of 15 mL with deionized water. Instrumental parameters for As, Cr, Cd and Ag determination by ICP OES followed established protocols (Oliveira et al., 2018). In this way, under robust conditions, the selection of the optimal wavelengths, free from spectral interferences for elemental determination by ICP OES, was carried out through scans across a wide wavelength range of the digested sample solution. These were then compared with scans obtained using the reference analytical solution, a multi-element solution containing 10 mg L^{-1} (As, Cr, Cd and Ag) in 0.1 % (v v^{-1}) HNO_3. The highest signal noise ratio and the non-overlapping of spectral lines were the criteria for choice of wavelengths used for elemental determination. Titrisol standard solutions (Merck, Darmstadt, Germany) of 1000 mg L^{-1} for As (As_2O_5), Cr ($CrCl_3$), Cd ($CdCl_2$), and Ag ($AgNO_3$), were used to prepare reference analytical solutions for calibration purposes, with concentrations ranging from 0.1 to 20 mg L^{-1} in 0.1 % (w v^{-1}) HNO_3. The instrumental conditions for elemental determination and characteristic parameters of the analytical calibration curves, such as linear range, coefficient of determination (R2), limit of detection (LOD), and limit of quantification (LOQ) are provided in the supplementary data (Table 2 and Table S1). LODs were determined based on the background equivalent concentration (BEC) and the signal-to-background ratio (SBR). The SBR was calculated using the formula: (I standard —I blank)/I blank, where I standard is the emission intensity of a reference solution, and I blank is the emission intensity of the blank. The BEC was calculated using the formula: BEC = C standard/SBR; where C standard is the concentration of the reference solution. The LOD was then calculated as: LOD (3 x BEC x RSD)/100 where the relative standard deviation (RSD) was derived from ten measurements of the analytical blank. LOQ was calculated as 3.3 times the LOD (Da Silva et al., 2007). The LOD and LOQ values, expressed as mass fractions (μg g^{-1}), were calculated considering a digested sample mass of 150 mg and a final volume of 15 mL. For the determination of LOD and LOQ values a multi-elemental reference solution containing As, Cr, Cd and Ag (5 mg L^{-1}) was used. The digested samples were measured in triplicate and

Table 2
Analytical method characteristics for elemental determination by ICP OES.

Element	Linear Range ($mg\ L^{-1}$)	R^2	Sensitivity	*LOD ($mg\ L^{-1}$)	**LOQ ($mg\ L^{-1}$)	*LOD ($\mu g\ g^{-1}$)	**LOQ ($\mu g\ g^{-1}$)	Recovery (%)
As	0.1–20	1.0000	279	0.009	0.0287	0.86	2.87	101
Cr	0.1–20	1.0000	790	0.001	0.0038	0.11	0.38	95
Cd	0.1–20	0.9999	541	0.005	0.0166	0.50	1.66	97
Ag	0.1–20	0.9999	438	0,003	0.0091	0.27	0.91	90

* LOD: Limit of Detection
** LOQ: Limit of Quantification

chemical interferences in the As, Cr, Cd and Ag determination by ICP OES were investigated through addition and recovery test. To this end, the samples were spiked with As, Cr, Cd, and Ag (1 $mg\ L^{-1}$) and recovery percentages for the target elements are also shown in Table 2. The recovery results ranged from 90 % to 101 %, indicating no matrix effects in elemental determination by ICP OES (FDA, 2020). According to the U.S. Food and Drug Administration's Elemental Analysis Manual for Food and Related Products, regarding elemental determination by spectrometric methods and the assessment of matrix-induced interference using fortified analytical solutions (FAS), which are spiked with the analyte prior to instrumental analysis, the control limits typically specify an FAS recovery of 100 ± 10 % (FDA, 2020). Mercury (Hg) and lead (Pb) mass fractions were measured in oyster samples using Atomic Absorption Spectrometry (AAS), a well-known selective technique for element determination, with a great freedom of interferences with its electrothermal (graphite furnace) mode of atomization (Welz & Sperling, 1999). Approximately 0.2 g of each sample was weighed into perfluoroalkoxy vials and digested in 10 mL of concentrated HNO_3 (P.A. grade) in a microwave oven (CEM MARS6), using the equipment's Animal Tissue program. Certified reference materials were digested and analyzed alongside the samples to ensure quality control (Table S2). After cooling, the digested samples were diluted to a total volume of 50 mL and analyzed. Hg concentrations were determined using Cold Vapor Atomic Absorption Spectrometry (CV-AAS) on a Perkin Elmer FIMS. At the same time, Pb levels were quantified via Electrothermal Atomic Absorption Spectrometry (ET-AAS) on a Perkin Elmer AAnalyst 800 spectrometer. In the CV AAS method, Hg is withdrawn from the sample solution as metallic mercury (Hg^0) with a argon flux while in the ET-AAS method, appropriate pyrolysis and atomization programs are used. Both steps avoid completely or minimize potential matrix interferences. Calibration curves were prepared fresh daily by diluting Spex Certiprep standard stock solutions, and calibration and reagent blanks were included for accurate mass fraction determination. Recovery results obtained on reference materials for Hg were 84 % and 108 % for IPEN MT-1 (Mussel Tissue) and CO-1 (Fish Tissue), respectively; and for Pb recovery results were 110 % and 101 % for IPEN MT-1 (Mussel Tissue) and MPH2 (Mixed Polish Herbs) respectively. To express the results on a wet weight basis, it was assumed that the dry weight represents 10 % of the wet weight.

2.4. Whole genome sequencing (WGS) of WHO critical priority bacterial pathogens

Genomic DNA from ESBL-producing Enterobacterales isolates was extracted and used to construct a paired-end library (150 bp), sequenced on the NextSeq platform (Illumina). The resulting short-read sequences were quality-trimmed using Trimmomatic v.0.39 (https://github.com/usadellab/Trimmomatic). De novo assembly was performed with Unicycler v.0.5.0 (https://github.com/rrwick/Unicycler). Species identification was carried out using the Type Strain Genome Server (https://tygs.dsmz.de/) and MLST (https://github.com/tseemann/mlst). For *Klebsiella pneumoniae* complex strains, further analysis of species, sequence type (ST), K-locus, and the outer core of lipooligosaccharide (OCL) was performed using Kleborate and Kaptive (https://github.com/klebgenomics/Kleborate; https://kaptive-web.erc.monash.edu/). For *Escherichia coli*, H and O antigens were determined using ECtyper (https://github.com/phac-nml/ecoli_serotyping). Virulence factors and plasmid replicons were screened using ABRicate v1.0.1 (https://github.com/tseemann/abricate) against the Virulence Finder and PlasmidFinder databases (https://www.mgc.ac.cn/VFs/; https://cge.food.dtu.dk/services/PlasmidFinder/). The presence of antibiotic resistance, heavy metal, and virulence genes was predicted using AMRFinderPlus (https://github.com/ncbi/amr). To determine the chromosomal or plasmid location of resistance genes, MOB-suite was employed (https://github.com/phac-nml/mob-suite). A minimum threshold of 90 % nucleotide identity and gene coverage was applied to ensure the accuracy of predictions. WGS data are available at NCBI BioProject: PRJNA1149029 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1149029).

2.5. Phylogenetic analysis

Comparative genomic analyses were conducted for *Klebsiella pneumoniae* MUC-SAN ST307, *Klebsiella quasipneumoniae* KQ-FLOR ST526, and *Citrobacter telavivensis* CIT-FLOR. To assemble a comprehensive dataset, global genome sequences were accessed from GenBank (retrieved January 2025) using taxon-specific identifiers: *K. pneumoniae* (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=573), *K. quasipneumoniae* (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=1463165), and *C. telavivensis* (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=2653932). Multilocus sequence typing (MLST) was applied to assign sequence types (STs) using MLST v2.23.0 (https://github.com/tseemann/mlst). For *Escherichia coli* FLOR1-OMV ST23 and CRO1-OMV ST38, South American genomes were acquired from the EnteroBase database (https://enterobase.warwick.ac.uk/species/ecoli/upload_reads) and classified according to the Achtman MLST scheme. A global dataset was constructed comprising 4*C. telavivensis* genomes, 44 *K. quasipneumoniae* ST526 genomes, and 47 *K. pneumoniae* ST340 genomes from South America. Additionally, South American subsets of *E. coli* included 28 genomes of ST23 and 122 genomes of ST38. These genomic datasets were subsequently utilized for phylogenetic analyses based on core genome alignments. Reference genomes of *E. coli* K12 (NCBI Reference Sequence: NC_000913.3), *Klebsiella pneumoniae* subsp. *pneumoniae* MGH78578 (NCBI Reference Sequence: CP000647.1), and *K. quasipneumoniae* FDAARGOS1503 (NCBI Reference Sequence: NZ_CP083633.1) strains were added to enhance the phylogenetic analysis. Genome annotations were performed using Prokka (https://github.com/tseemann/prokka). Comparative core genome multilocus sequence typing (cgMLST) was conducted via Panaroo, operating in "strict" mode with a core threshold of 1.0. This analysis generated concatenated core-genome alignments comprising 4198 genes (3,838,558 bp in length) for *C. telavivensis*, and 3546 genes (3,244,170 bp) for *K. pneumoniae* ST307 and *K. quasipneumoniae* ST526. For *E. coli* ST23 and ST28, concatenated core-genome alignments included 3573 genes (3,033,429 bp). Phylogenetic trees were constructed with maximum likelihood in RAxML-NG v1.2.2 (https://github.com/amkozlov/raxml-ng), employing the GTR + G substitution model. Node support was evaluated with 100 bootstrap replicates to ensure

branch reliability (option "–bs-trees 100"). Single nucleotide polymorphisms (SNPs) were identified using SNP-sites (https://github.com/sanger-pathogens/snp-sites), and a SNP distance matrix was generated with snp-dists v0.8.2 (https://github.com/tseemann/snp-dists). Finally, tree visualization and annotation were carried out using iTOL v6 (https://itol.embl.de/).

2.6. Conjugation assays

Conjugation assays were conducted with minor modifications as previously described (Møller et al., 2017). ESBL-positive isolates served as donor strains, while *E. coli* C600 (streptomycin-resistant) acted as the recipient strain. Bacterial strains were first streaked onto LB agar plates and subsequently inoculated in LB broth for overnight growth. Overnight cultures were diluted 1:100 in fresh LB broth and incubated at 37 °C for 3 h. The donor and recipient cultures were then mixed at a 1:3 ratio, and 40 μL of the mixture was spotted onto 0.22 μm sterile membrane filters placed on LB agar plates, followed by incubation at 37 °C for 18 h. After incubation, the conjugation mixture was retrieved from the filters using sterile saline. A 50 μL aliquot from each dilution was then evenly spread onto selective LB agar plates supplemented with antimicrobials for screening. Each dilution was plated in triplicate to ensure reproducibility. LB agar supplemented with ceftriaxone (2 μg/mL) and streptomycin (250 μg/mL) was used to select ESBL-bearing transconjugants. The presence of resistance genes in transconjugants was verified by PCR and assessment of resistance phenotypes. Primers and PCR conditions for bla_{SHV-5} and *tetA* were performed as previously described (Chang et al., 2001; Cho et al., 2019). The conjugation frequency was determined by calculating the ratio of transconjugant colonies to recipient cells.

2.7. In vivo virulence assays of WHO critical priority Enterobacterales using the Galleria mellonella infection model.

The virulence potential of bacterial strains was assessed using the *Galleria mellonella* infection model. Groups of ten larvae, weighing approximately 250–350 mg, were inoculated with 10^5 CFU of *E. coli* or 10^6 for *Klebsiella pneumoniae,* and survival was monitored hourly for 96 h. Hypervirulent controls consisted of *E. coli* strain MNEC RS218 (hypervirulent meningitis/sepsis-associated K1) and *K. pneumoniae* K1 (hypervirulent liver abscess-associated K1) (Dantas et al., 2024; Moura et al., 2018). To ensure reproducibility, two technical replicates were performed. To verify the infection dose, the inoculum was serially diluted and plated on MacConkey agar to ensure accurate colony enumeration. Survival curves were plotted using the Kaplan-Meier method with GraphPad Prism software, version 8.3.0.

2.8. Statistical analysis

Statistical analyses were conducted using Phyton v. 3.13.2, with data visualization performed through matplotlib library. The Shapiro-Wilk test was employed to evaluate the normality of the datasets. Differences between groups in terms of virulence and resistance gene counts per strain were assessed using Mann-Whitney test using SciPy library. A *p*-value of less than 0.05 was considered statistically significant. Furthermore, heavy metal quantification was performed in triplicate, and the results were reported as mean values accompanied by standard deviations.

3. Results

3.1. Quantification of toxic heavy metals reveals high bioaccumulation of As in RTE oysters

Higher concentrations of total As (> 1 mg/kg) were detected in RTE raw oysters from markets M3, M4, and M5 (Table 3). In oysters from market M3, a concentration of 1.86 ± 0.31 mg/kg wet weight of total As was found in oysters collected during the summer season of 2023. In contrast, in market M4, concentrations of 1.01 mg/kg wet weight of total As were detected in oysters collected during the winter season of 2022, and 1.95 ± 0.05 mg/kg wet weight in oysters collected during the summer season of 2023. Finally, in oysters from market M5, a concentration of 1.57 ± 0.01 mg/kg wet weight of total As was detected during the summer season of 2023. Moreover, total Cd concentration (1.6 ± 0.14 mg/kg wet weight) was detected in oysters from market M1 in oysters collected during the summer season of 2023. Concentrations of total Pb and Cr were detected (0.07 to 0.9 mg/kg). Finally, for Hg quantification, all samples were below the limit of detection (< 0.0027 mg/kg) by using AAS method.

3.2. Presence of WHO critical priority Enterobacterales producing ESBLs in RTE oysters containing high levels of As

Initially, twenty-five antibiotic-resistant bacterial strains were isolated from oysters collected from the 5 markets, spanning the winter of 2022 to the summer of 2023 (Table 1). Notably, none of the isolates belonged to Gram-positive bacterial species or *Vibrio* genus. In the winter season of 2022, antibiotic-resistant *Escherichia coli*, *Aeromonas* spp., *Proteus* spp., *Klebsiella pneumoniae*, and *Citrobacter* spp. were isolated from oysters collected from M1, M2 and M4 markets (Table 1). Strikingly, two ceftriaxone-resistant *Klebsiella pneumoniae* isolated from oysters purchased from M2 (strain MUC-SAN) and M4 (strain KQ-FLOR) markets, and a *Citrobacter* spp., (strain CIT-FLOR) recovered from oysters from the M4 market exhibit a positive ESBL phenotype. On the other hand, in the summer season of 2023 antibiotic-resistant *E. coli*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Pseudomonas otitidis*, *Aeromonas* sp., *Comamonas aquatica* and *Proteus* spp. were isolated from oysters purchased from all markets, of which two strains of *E. coli* (strains FLOR1-OMV and CRO1-OMV) isolated from oysters from the M2 market were characterized as ESBL producers.

Table 3
Concentration of heavy metals in ready-to-eat oysters purchased from markets in Brazil.

Market ID	State, City	Specie	Season/year	Total concentration of heavy metals (mg/kg) wet weight				
				As	Ag	Cd	Cr	Pb
M1	São Paulo, Cananéia	*C. brasiliana*	Winter 2022	0.44 ± 0.02	0.51 ± 0.06	0.47 ± 0.03	< DL	0.60 ± 0.06
			Summer, 2023	0.49 ± 0.01	0.74 ± 0.03	1.6 ± 0.14	0.07 ± 0.002	0.54 ± 0.04
M2	São Paulo, Santos	*C. brasiliana*	Summer, 2023	0.74 ± 0.02	0.90 ± 0.19	< DL	0.07 ± 0.003	0.92 ± 0.02
M3	São Paulo, São Paulo	*C. gigas*	Winter 2022	0.91 ± 0.03	0.42 ± 0.02	< DL	0.09 ± 0.001	0.43 ± 0.03
			Summer, 2023	1.86 ± 0.31	0.45 ± 0.16	< DL	0.23 ± 0.04	0.43 ± 0.05
M4	Santa Catarina, Florianópolis	*C. gigas*	Winter 2022	1.01 ± 0.01	0.99 ± 0.26	0.44 ± 0.02	0.11 ± 0.01	0.73 ± 0.03
			Summer, 2023	1.95 ± 0.05	<DL	<DL	0.44 ± 0.01	0,65 ± 0,01
M5	São Paulo, Peruíbe	*C. brasiliana*	Summer 2023	1.57 ± 0.01	0.83 ± 0.04	0.26 ± 0.02	0.12 ± 0.01	0.90 ± 0.04

<DL; less to the detection limit

3.3. Antimicrobial resistance and heavy metal tolerance profiles of ESBL-positive Enterobacterales carried by RTE oysters

In brief, a total of 5 ESBL-positive Enterobacterales displaying a multidrug-resistant (MDR) profile to clinically relevant beta-lactam, quinolone, trimethoprim/sulfamethoxazole, tetracycline, and aminoglycoside antibiotics (Magiorakos et al., 2012), were recovered from oysters obtained from M2 and M4 markets (Table 4). Noteworthy, all oyster-derived isolates exhibited a multi-metal tolerance profile, defined as tolerance to ≥3 heavy metal compounds, with minimum inhibitory concentrations (MICs) for As, Ag, Cu, Hg, Co, and Cr of ≥1024, ≥ 8, 2048, ≥ 1, ≥ 512, and 128 μg/mL, respectively.

3.4. WGS analysis reveals international clones of WHO critical priority Enterobacterales harboring a wide resistome in RTE oysters and emergence of Citrobacter telavivensis in seafood

The WGS analysis of ESBL-producing Enterobacterales confirmed *Citrobacter telavivensis*, global *K. pneumoniae* sequence type (ST) ST307, the emergent *K. quasipneumoniae* ST526, and *E. coli* belonging to the international clones ST23 (O78:H9) and ST38 (O88:H18), in RTE raw oysters exhibiting high level of As bioaccumulation (Table 4). Antibiotic and heavy metal resistance genes (ARGs and HMRGs) analysis of *Citrobacter telavivensis* strain CIT-FLOR predicted $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{SED-like}}$ β-lactamase genes, and *arsABCDER* (As tolerance), *merBDR* (Hg tolerance), and *terDWZ* (Te tolerance) genes, along with plasmid incompatibility groups IncHI2A, IncHI2 (Fig. 1). On the other hand, the *K. pneumoniae* strain MUC-SAN belonged to ST307, and it carried $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{SHV-28}}$, $bla_{\text{TEM-1}}$ β-lactamase genes, *arsABCDER* and *pcoES* (Cu tolerance) genes, and IncFIB(K)_1_Kpn3 plasmid incompatibility groups. The *K. quasipneumoniae* strain KQ-FLOR belonged to ST526, harboring the beta-lactam resistance genes $bla_{\text{OKP-A-11}}$, $bla_{\text{SHV-5}}$, and *arsABCDER, silABCEFPRS* (Ag tolerance), *pcoABCDRS* and *fieF* (Fe, Cd and Zn tolerance) genes, along with plasmid incompatibility groups IncFIB(pKPHS1), IncFIA(HI1), IncFIB(K), IncN, and Col440I. For this strain, conjugation experiments showed successful transfer of $bla_{\text{SHV-5}}$ and *tetA* genes conferring resistance to third-generation cephalosporins (CRO, CAZ, CPM) and tetracyclines, respectively, to *E. coli* C600 recipient strain, as confirmed by PCR. In this respect, the transfer frequency obtained was 5.2×10^{-1} transconjugants/recipient. Finally, while the *E. coli* strain FLOR1-OMV belonged ST23, harboring the $bla_{\text{CTX-M-55}}$ ESBL gene, *arsBCR, fieF* genes, and plasmid incompatibility groups IncFIB (AP001918), IncR, IncX4, and IncFII(pHN7A8); the *E. coli* strain CRO1-OMV belonged to ST38 and harbored the ESBL resistance genes $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$, *arsBCR, fieF*, and plasmid incompatibility groups IncFII and ColRNAI.

3.5. Phylogenomic analysis confirms clonal relationship of Enterobacterales carried by RTE oysters with human-associated E. coli and Klebsiella spp. circulating in South America

The *C. telavivensis* strain CIT-FLOR, isolated from ready-to-eat oysters, in this study, represents the first report of this species in a non-clinical setting. This strain carried the $bla_{\text{CTX-M-15}}$ ESBL, highlighting its potential as an emerging critical priority pathogen (Fig. 2). Phylogenomic cgMLST comparative analysis with 3 publicly available *C. telavivensis* genomes revealed 30,542 SNPs difference with the *C. telavivensis* strain 6106 (GenBank: WHIY00000000.1) isolated in 2010 from a hospitalized patient in Tel-Aviv, Israel (Table S3 and Table S8). Additionally, global metadata of *C. telavivensis* revealed the presence of heavy metal operons *ars*, *sil*, *pco*, and *ter* in 100, 25, 25, and 25 % of analyzed genomes, respectively (Fig. 3).

Based on 47 high-quality publicly available South American genomes of *K. pneumoniae* ST307, most originating from human hosts, and from environmental and animal sources (including mussels, oysters, and birds), cgMLST analysis revealed close genomic relatedness (54 SNPs difference) of *K. pneumoniae* ST307 strain MUC-SAN with the *K. pneumoniae* strain PEROUPCH20K (GenBank: JAGGIZ000000000.1), isolated from wild boar (*Sus scrofa*) in Peru, in 2017 (Fig. 4, Table S4 and Table S9). Interestingly, all South American *K. pneumoniae* ST307 share the KL102-O1/O2v2 locus (Fig. 4). On the other hand, *ars*, *sil*, *pco*, *ter*, *ncr*, and *mer* heavy metal operons were detected in 89, 79, 79, 17, 2, and 6 % of South American ST307 clones, respectively (Fig. 3).

Analysis of 44 global genomes of *K. quasipneumoniae* ST526 revealed seven KL-types (KL70, KL113, KL135, KL137, KL141, KL172, KL176) and six OL-types (O1/O2v3, O3/O3a, O3b, O4, O12, OL102), with isolates originating from human and non-human sources, including sewage and sediments (Fig. 4). Specifically, the strain KQ-FLOR (ST307:KL135:O3/O3a serotype) isolated from oysters, in this study, was closely related (216 SNPs) to a *K. quasipneumoniae* ST526 (strain EW669; GenBank: JBAFZS000000000.1) isolated from aquatic environment in Brazil, in 2020 (Table S5 and Table S9). Heavy metal operons *ars*, *fie*, *sil*, *pco*, *ter*, *ncr*, and *mer* were detected in 45, 100, 66, 61, 23, 18, and 7 % of global *K. quasipneumoniae* ST526 genomes, respectively. On the other hand, the $bla_{\text{SHV-5}}$ ESBL gene was exclusively identified in the oyster KQ-FLOR strain, suggesting a novel critical priority lineage of

Table 4
Antimicrobial resistance and heavy metal tolerance profiles of ESBLs-positive Enterobacterales isolated from RTE oysters collected from markets M2 and M4 in Brazil.

Strain	Isolate, ST [a], serotype [b]	Origin (year)	Market ID	Antibiotic resistance profile	Heavy metal resistance profile (MIC μg/mL)
MUC-SAN	*K. pneumoniae* 307 KL102 O1/O2v2	*C. brasiliana* (2022)	M2	ATM, AMP, AMO, AMC, TIC, CAZ, CPM, CRO, CTX, PIP, SPT, CIP, NAL, SUT	Arsenic (2048), Copper (2048), Cobalt (512), Chromium (128), Silver (16), Mercury (2)
KQ-FLOR	*K. quasipneumoniae* 526 KL135 O3/O3a	*C. gigas* (2022)	M4	ATM, AMP, AMO, TIC, CAZ, CPM, CRO, CTX, PIP, ETP, CFO, TET, DO, CIP, SUT	Arsenic (1024), Copper (2048), Cobalt (512), Chromium (128), Silver (16), Mercury (4)
CIT-FLOR	*Citrobacter telavivensis*	*C. gigas* (2022)	M4	ATM, AMP, AMO, TIC, CAZ, CPM, CRO, CTX, PIP, GEN, CIP, NAL, SUT	Arsenic (4096), Copper (2048), Cobalt (1024), Chromium (128), Silver (8), Mercury (1)
CRO1-OMV	*E. coli* 38 O88:H18	*C. brasiliana* (2023)	M2	ATM, AMP, AMO, AMC, TIC, CAZ, CPM, CRO, CTX, PIP, CFO, C, FLOR, CIP, NAL	Arsenic (1024), Copper (2048), Cobalt (1024), Chromium (128), Silver (8), Mercury (1)
FLOR1-OMV	*E. coli* 23 O78:H9	*C. brasiliana* (2023)	M2	AMP, AMO, TIC, CRO, CTX, PIP, SPT, TET, DO, C, FLOR, FOS, SUT	Arsenic (1024), Copper (2048), Cobalt (1024), Chromium (128), Silver (8), Mercury (1)
E. coli ATCC® 25922™	–	*Control*	–	*	Arsenic (16), Copper (1024), Cobalt (512), Chromium (128), Silver (4), Mercury (1)
K. pneumoniae KPN535	–	*Control*	–	CPM, CRO, CTX, AMI, GEN, TET, SUT	Arsenic (2048), Copper (2048), Cobalt (512), Chromium (128), Silver (16), Mercury (16)

[a] ST, sequence type predicted by MLST 2.0 (https://cge.food.dtu.dk/services/MLST/).
[b] Serotype predicted using Kleborate for *Klebsiella* genus and ECtyper for *E. coli* strains. Resistance profile by disk-diffusion. ATM; aztreonam, AMP; ampicillin, AMO; amoxicillin, AMC; amoxicillin-clavulanic, TIC; ticarcillin, CAZ; ceftazidime, CPM; cefepime, CRO; ceftriaxone, CTX; cefotaxime, PIP; piperacillin, ETP; ertapenem, GEN; gentamicin, SPT; streptomycin, CIP; ciprofloxacin, CFO; cefoxitin, NAL; nalidixic acid, TET; tetracycline, DO; doxycycline C; chloramphenicol, FLOR; florfenicol, FOS; fosfomycin, SUT; sulfamethoxazole/trimethoprim.
* Susceptible to all antibiotics tested.

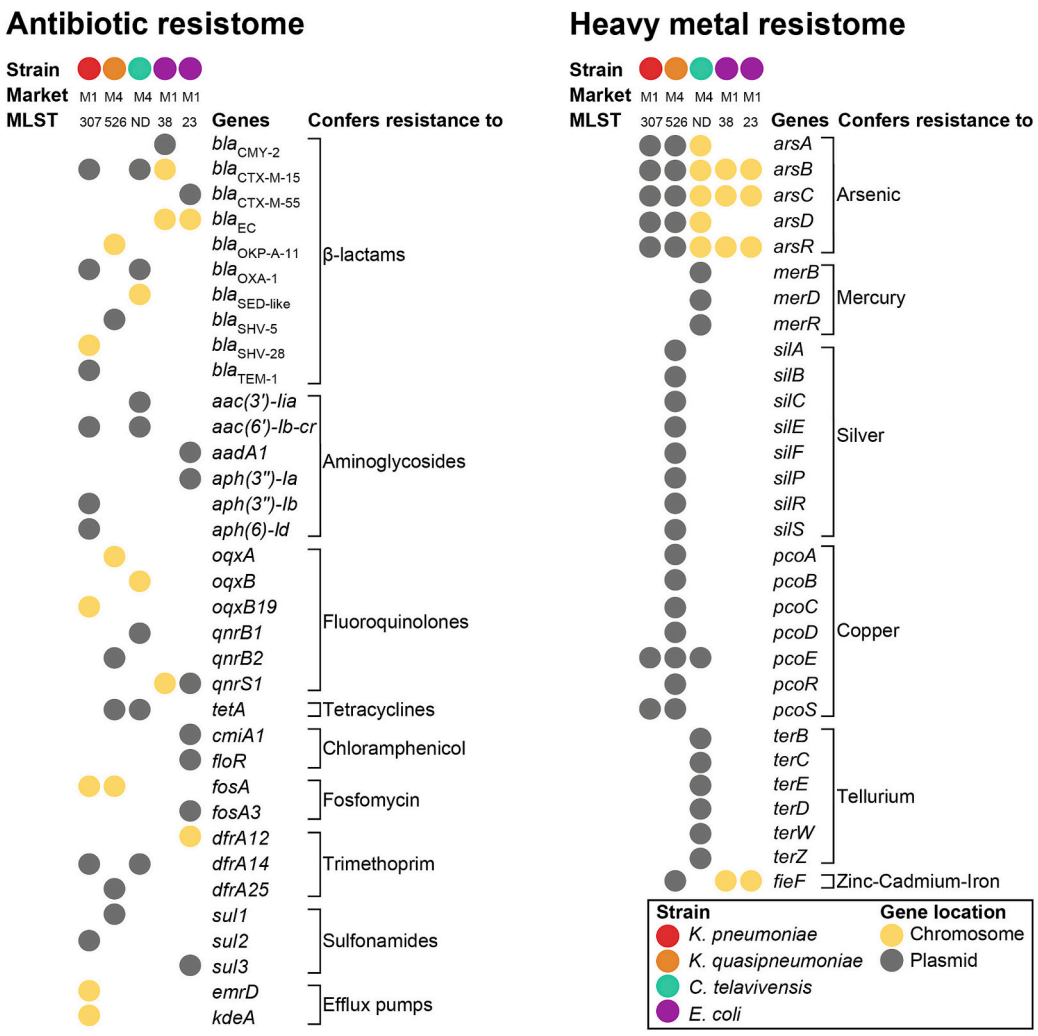

Fig. 1. Antibiotic and heavy metal resistome of ESBLs positive *Citrobacter telavivensis* CIT-FLOR, *Klebsiella pneumoniae* MUC-SAN, *Klebsiella quasipneumoniae* KQ-FLOR, *E. coli* CRO1-OMV and FLOR1-OMV isolated from ready-to-eat oysters.

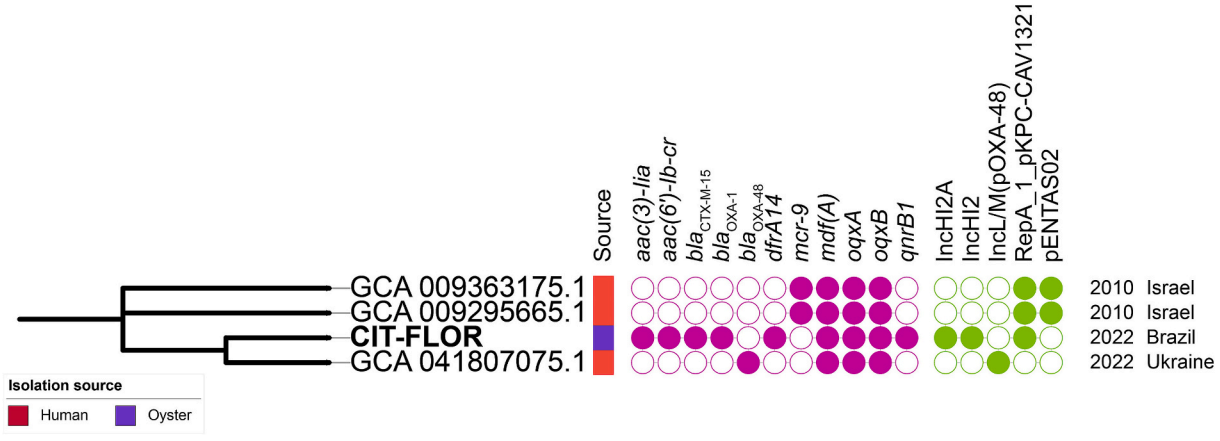

Fig. 2. cgMLST analysis and heatmap depicting the beta-lactam resistome and plasmidome, isolation source and collection year of strain CIT-FLOR alongside publicly available *Citrobacter telavivensis* genomes isolated at the human-animal interface worldwide (2010–2022). A maximum-likelihood phylogenetic tree was constructed based on a filtered core genome alignment generated using the Panaroo software, consisting of 4198 genes (3,838,558 bp) from four *C. telavivensis* strains. The antimicrobial resistance genes *aac(3)-Iia*, *aac(6')-Ib-cr* (aminoglycoside resistance), $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$ (β-lactam resistance), *dfrA14* (trimethoprim resistance), *qnrB1* (quinolone resistance), and the plasmid incompatibility groups IncHI2A and IncHI2, were exclusively identified in strain CIT-FLOR, which was isolated from ready-to-eat oysters in Florianopolis, Brazil. Phylogenetic tree visualization was performed using iTOL v6.

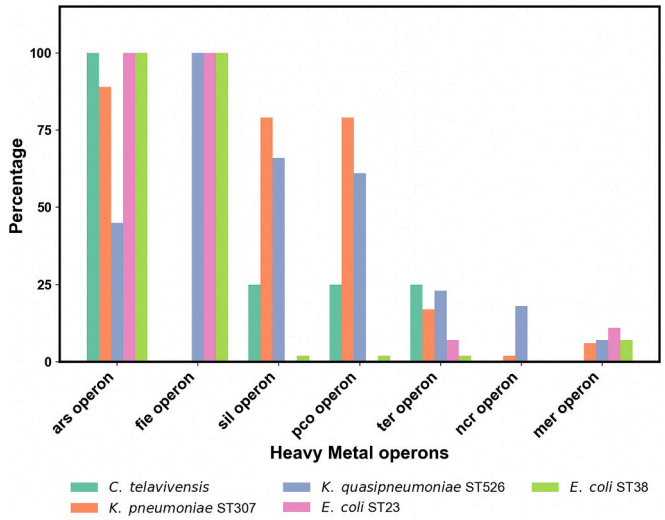

Fig. 3. Proportion of heavy metal operons across global and South American genomes. The analysis includes global genomes of *Citrobacter telavivensis* ($n = 4$) and *Klebsiella quasipneumoniae* ST526 ($n = 44$), as well as South American genomes of *Klebsiella pneumoniae* ST307 ($n = 47$), *Escherichia coli* ST23 ($n = 28$), and *E. coli* ST38 ($n = 123$). Only complete operons were considered, categorized as follows: arsenic operon (*arsRBC*), copper operon (*pcoABCDRSE*), mercury operon (*merRTPA*), silver operon (*silESRCFBA*), tellurite operon (*terBCDE*), iron and zinc operon (*fieF*), and nickel and cobalt operon (*ncrABCY*). The most prevalent operon, *arsRBC*, was detected in 100 % of the analyzed genomes of *Citrobacter telavivensis*, *Escherichia coli* ST23, and *E. coli* ST38. It was also identified in 89 % of *Klebsiella pneumoniae* ST307 genomes and 45 % of *Klebsiella quasipneumoniae* ST526 genomes.

K. quasipneumoniae emerging in Brazil.

For *E. coli* ST23, phylogenomic analysis of 28 South American genomes from human and non-human hosts revealed that the oyster strain FLOR1-OMV was closely related (131 SNPs difference) to the *E. coli* strain 97–1-cefta (GenBank: ABKFVS000000000.1) isolated from meat in Chile, in 2019 (Table S6 and Table S10). Among, Latin American *E. coli* ST23, four serotypes could be identified (i.e., O8:H9, O85:H21, O78:H9, O32/O8:H9), with CTX-M-15/CMY-2-co-producing *E. coli* CRO1-OMV belonging to the O78:H9 serotype (Fig. 5). Operons *ars*, *fie*, *ter*, and *mer* were detected in 100, 100, 7, and 11 % of South American *E. coli* ST23 genomes.

In silico analyses of 123 South American *E. coli* ST38 genomes from human, environmental, and animal sources, revealed 11 serotypes, including O1:H15, O2/O50:H30, O7:H15, O7:H18, O86:H18, O88:H18, O102:H6, O153:H2, O153:H9, O153:H18, O153:H30 (Fig. 5). The oyster strain CRO1-OMV was related (2448 SNPs difference) to an environmental *E. coli* ST38 strain LSI_129 (Enterobase: ESC_BB1213AA) isolated in Ecuador, in 2020 (Table S7 and Table S10). Heavy metal operons *ars*, *fie*, *sil*, *ter*, *pco*, and *mer* were detected in 100, 100, 2, 2, 2, and 7 % of South American *E. coli* ST38 genomes, respectively.

3.6. Distribution and comparison of antimicrobial resistance and virulence genes of Enterobacterales carried by RTE oysters

Following the phylogenetic analysis of ESBL-positive *Enterobacterales* strains, an in-depth investigation was conducted to determine the number of antibiotic resistance genes in *Klebsiella pneumoniae* ST307 ($n = 47$, South America), *Escherichia coli* ST23 ($n = 28$, South America), *E. coli* ST38 ($n = 122$, South America), *K. quasipneumoniae* ST526 ($n = 44$, global), and *Citrobacter telavivensis* (n = 4, global). The results indicate that β-lactamase and aminoglycoside resistance genes are the most abundant resistance determinants across these groups (Fig. 6A). To further investigate resistance gene distribution, we compared the number of β-lactamase and aminoglycoside resistance genes per strain within each sequence type. The analysis revealed that the South American *K. pneumoniae* ST307 group harbored significantly more β-lactamase resistance genes (mean: 4.5 ± 1.1 genes per strain, $P < 0.001$) than all other groups analyzed (Fig. 6B). Similarly, *K. pneumoniae* ST307 from South America carries significantly more aminoglycoside resistance genes (mean: 4.2 ± 1.2 genes per strain, $P < 0.001$) compared to the other groups (Fig. 6C). In addition, to assess the virulence potential of *E. coli*, we compared the number of virulence genes between South American strains belonging to ST23 and ST38. The results show that *E. coli* ST38 harbors significantly more virulence genes than ST23 ($P < 0.001$), suggesting a higher virulence potential for this sequence type (Fig. 6D).

3.7. Virulome and virulent potential of ESBL-producing Escherichia coli clones ST23 and ST38, and K. pneumoniae ST307 carried by RTE oysters

The virulome of the CTX-M-15-positive *E. coli* ST38 (CRO1-OMV strain) was associated with the presence of adherence (*fimABCDEFGHI*, *fdeC*), invasion (*aslA*), protectins/serum resistance (*kpsDM*, *ompA*), iron uptake (*shuATX*, *chuSUVWY*, *entABCDEFS*, *fepABCDG*, *fes*), and secretion system (*espL1*, *espL4*, *espR1*, *espX1*, *espX4*, *espX5*, *espY1*, *espY3*, *espY4*, *gspCDEFGHIJKLM*) genes. On the other hand, the CTX-M-55-positive *E. coli* ST23 FLOR1-OMV carried adherence (*csgBDFG*, *fimABCDEFGHI*, *fdeC*, *yagVWXYZ*, *ecpABCDE*, *ykgK*, *ecpR*), protectins/serum resistance (*ompA*), iron uptake (*iroBCDEN*, *iucABCD*, *iutA*, *fepABCDG*, *entABCDEFS*, *fes*), and secretion system virulence genes (*espL1*, *espR1*, *espX1*, *espX4*, *espX5*, *gspEFGHIJKLM*). Both *E. coli* strains displayed a highly virulent behavior with higher mortality rates of *G. mellonella* larvae than the virulent control *E. coli* MNEC-K1, killing 100 % of the larvae in less than 12 h (Fig. 7).

Although, the *Klebsiella pneumoniae* ST307-KL102 (strain MUC-SAN) showed a mucoid phenotype (string test ≥5 mm), no virulence genes were identified. However, 75 % and 85 % larvae infected with MUC-SAN died at 12 and 96 h post-infection, respectively.

4. Discussion

Seafood consumption is associated with health benefits attributed to its nutritional composition, such as omega-3 fatty acids, and neuroprotective iodine and selenium. Indeed, some dietary guidelines recommend twice-weekly consumption of seafood for health benefits (Errickson et al., 2024). Unfortunately, edible bivalves, such as oysters, can accumulate organic and inorganic pollutants, including harmful heavy metals, resulting from marine contamination by industrial discharge and agricultural runoff (Seiler & Berendonk, 2012). Moreover, contamination of shellfish with foodborne pathogens can occur during handling, processing, or directly within the marine environment.

Recently, clinically relevant antimicrobial-resistant bacteria have begun to be detected in seafood (Mitchell et al., 2024), including oysters (Mohammed et al., 2024; Nasser et al., 2024), which is a critical issue, since many edible bivalves are commercialized as RTE foods, being prone to transmit antimicrobial-resistant bacteria and/or their resistance genes to humans. Recently, ESBL-producing critical priority *E. coli* were reported in oysters from Egypt. However, this study has not used genomic approaches or correlation with arsenic bioaccumulation (Mohammed et al., 2024), Some intrinsic aspects of bivalves such as filtration properties and bioaccumulation of heavy metals, such as arsenic, could contribute to selection of WHO critical-priority Enterobacterales, which have adapted to marine environments after their dissemination beyond hospital walls, because of anthropogenic pollution (Fernandes et al., 2020; Sellera et al., 2018; Tavares et al., 2020). Thus, since most microbiological studies in seafood has focused on foodborne pathogens, following current FAO recommendation, we have conducted a surveillance study to investigate WHO critical-priority antimicrobial-resistant Enterobacterales in RTE raw oysters, in Brazil. In this country, aquaculture practice represented revenue of U$ 1 billion

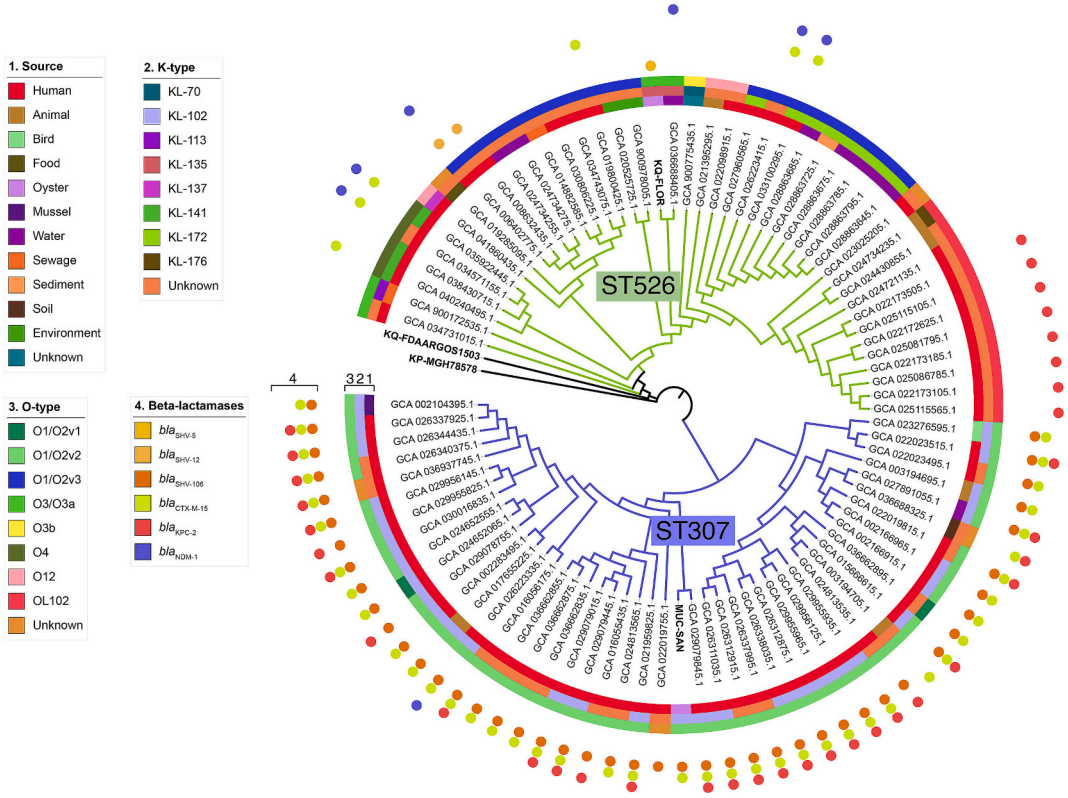

Fig. 4. cgMLST analysis and heatmap illustrating SHV variants, $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{NDM-1}}$ beta-lactamases, isolation sources, and K-locus and O-locus types of publicly available South American genomes of *Klebsiella pneumoniae* ST307 (blue clade) and global genomes of *Klebsiella quasipneumoniae* ST526 (green clade). A maximum-likelihood phylogenetic tree was constructed from a filtered core genome alignment generated using Panaroo software, encompassing 3546 genes (3,244,170 bp) for *K. pneumoniae* ST307 and *K. quasipneumoniae* ST526. From the inner to the outer circles: (1) KL-locus, (2) OCL-locus, (3) collection source, and (4) beta-lactamases and carbapenemases diversity. Notably, 100 % of the analyzed ST307 genomes from South America exhibited the presence of $bla_{\text{SHV-106}}$ and $bla_{\text{CTX-M-15}}$ β-lactamases. Conversely, the global distribution of ST526 revealed that $bla_{\text{SHV-5}}$ was exclusively identified in strain KQ-FLOR. Phylogenetic tree visualization was performed using iTOL v6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in 2019, which corresponded to a production of 800,000 t. In fact, mollusk farming is among the main sectors of aquaculture in this country, where oysters are largely consumed as seafood (Vieira et al., 2021).

In the oysters analyzed, we initially detected elevated concentrations of total As, compared to other potentially toxic metals (Ag, Cd, Cr, Hg, Pb), along with the identification of clinically relevant AMR bacterial pathogens, which led us to investigate the genomic background and its correlation with antimicrobial resistance, heavy metal tolerance and virulence potential, in order to warn of possible risks to human host, associated with seafood consumption. In our study, total As levels in raw oysters (*Crassostrea gigas* and *Crassostrea brasiliana*) ranged from 0.44 to 1.95 mg/kg. These values are consistent with previous reports: 0.45–3.0 mg/kg in *C. gigas* from Italy (Battistini et al., 2021), 5.44–9.56 mg/kg in *C. gigas* from Mexico (Bergés-Tiznado et al., 2013), and 0.55–2.0 mg/kg in *C. iredalei* from Malaysia (Affizah et al., 2009). In Brazil, the regulation of heavy metal concentrations in bivalve mollusks falls under the responsibility of ANVISA (National Health Surveillance Agency), which sets maximum limits of 1 mg/kg for total As, 2 mg/kg for Cd, 1.5 mg/kg for Pb, and 0.5 mg/kg for total Hg (ANVISA, 2022). In this context, our result showed that three markets (M3, M4 and M5) exceeded the As limits set by Brazilian regulations. Similarly, the European Union (EU) has established maximum permissible levels for Pb (1.5 mg/kg), Cd (1.0 mg/kg), and Hg (0.5 mg/kg) in bivalve mollusks (European Union (EU), 2023). According to the World Health Organization, the tolerable weekly intake of inorganic As is (0.015 mg/kg (bodyweight/week) (World Health Organization (WHO), 2011). The occurrence of any clinically relevant pathogen resistant to third-generation cephalosporins, in RTE oysters, should be considered abnormal and undesirable, becoming a public health risk that deserves continued surveillance. In this regard, the number of reports on critical-priority bacteria in seafood, in recent years, has increased highlighting the need for better management in the production systems.

Arsenic's toxicity depends on its chemical form: inorganic arsenic (iAs) is highly toxic, whereas organic forms, such as arsenobetaine (AsB), are generally non-toxic and not metabolized. In oysters and mussels, AsB often constitutes 60–90 % of total As, while iAs represents a minor fraction (Bergés-Tiznado et al., 2013; Moreda-Piñeiro et al., 2010). Additionally, an ICP-MS study identified ten As species in oysters, with organic As compounds predominating. AsB accounted for more than 90 % of the total organic As, while As(III) was generally undetected, and only trace levels of As(V) were present (Nam et al., 2015). Based on these findings, we suggest that a similar distribution should occur in the analyzed oyster samples, with most of the total As in the form of organic As, primarily arsenobetaine. While organic As, such as AsB, is considered less toxic, the potential for conversion to iAs by the gut microbiome raises concerns regarding the cumulative health impact of regular oyster consumption (Mukherjee et al., 2024). A limitation of our study is that the origin and location of these seafood supplies was not possible to obtain, which could be epidemiologically relevant to determine if the areas of provenance are contaminated by industrial, hospital, or other sources of microbial or chemical contamination. On the other hand, only total As concentrations were measured. Future research should employ advanced methodologies, such as High-Performance Liquid Chromatography coupled with Inductively Coupled Plasma Mass Spectrometry (HPLC–ICP-MS) and μ-X-ray absorption near-edge structure (μ-XANES) analysis, to quantify iAs levels accurately and better assess associated health risks.

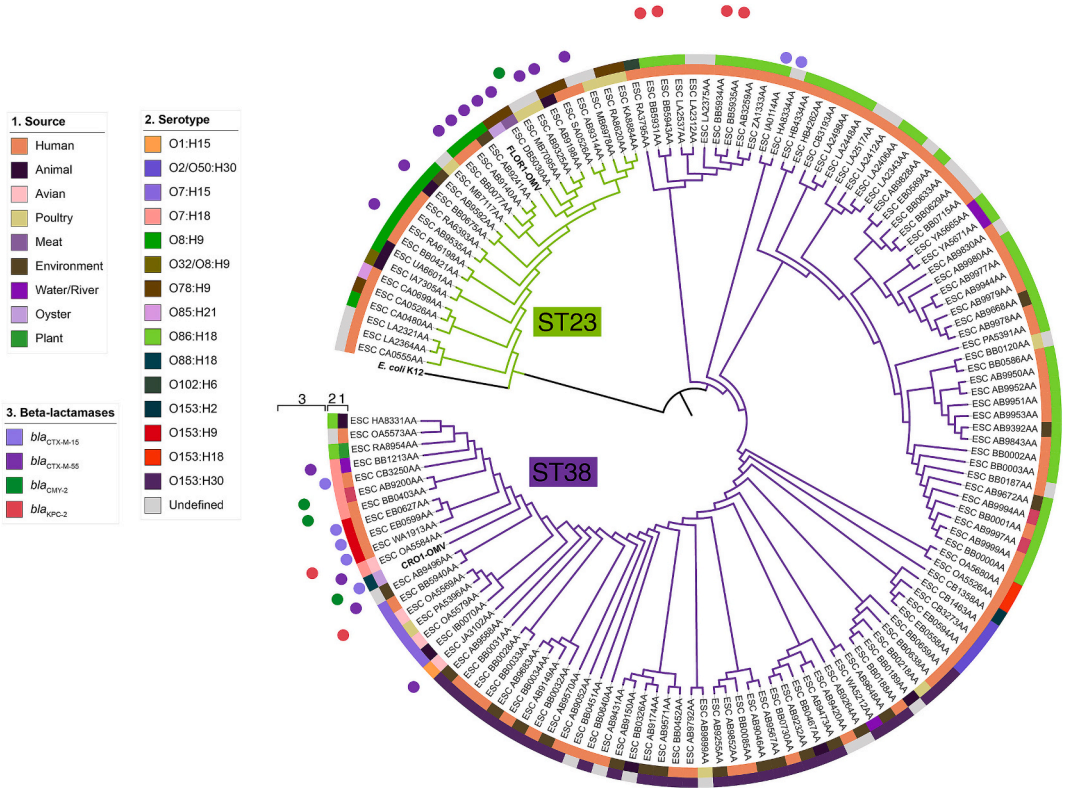

Fig. 5. cgMLST analysis and heatmap illustrating $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$, $bla_{\text{CMY-2}}$, and $bla_{\text{KPC-2}}$ beta-lactamases, isolation sources, and serotypes of publicly available South American genomes of *Escherichia coli* ST23 (green clade) and ST38 (blue clade). A maximum-likelihood phylogenetic tree was constructed based on a filtered core genome alignment generated using Panaroo software, comprising 3573 genes (3,033,429 bp) for ST23 and ST38. From the inner to the outer circles: (1) serotype, (2) isolation source, and (3) beta-lactamases and carbapenemases diversity. Notably, strain CRO1-OMV is located in a human-animal-environment subclade containing most beta-lactamases identified in ST38. Furthermore, CRO1-OMV was the only strain co-producing $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$ in South America. In contrast, strain FLOR1-OMV is located within an animal-associated subclade linked to $bla_{\text{CTX-M-55}}$. Phylogenetic tree visualization was performed using iTOL v6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Worryingly, the occurrence of WHO critical ESBL-producing Enterobacterales was confirmed in oysters containing high-level of total As. In this regard, a higher number of Gram-negative resistant strains of bacteria were observed in *C. brasiliana* than *C. gigas*, mainly in the summer period of sample collection (Table 1). This difference may reflect variations in the environmental conditions and handling practices associated with these products. Infections caused by multidrug-resistant (MDR) *E. coli* and *K. pneumoniae* often originate from asymptomatic gastrointestinal colonization in humans (Armand-Lefèvre et al., 2018; Denkel et al., 2020). Chronic carriers can transmit these pathogens to individuals in close contact (Hilty et al., 2012), emphasizing the public health risks associated with oyster consumption and the potential role of these shellfish in facilitating the spread of AMR microorganisms. Notably, the isolation of ESBL-producing strains from non-clinical settings demonstrates the capacity of oysters to harbor clinically significant AMR pathogens. Of epidemiological concern is the identification of international clones of human-associated *K. pneumoniae* ST307, and *E. coli* ST38 and ST23. *K. pneumoniae* ST307 is recognized globally as a public health concern due to its association with multidrug resistance and its ability to cause severe infections in both hospital and community environments (Peirano et al., 2020). Previous reports identified this sequence type in clams from Tunisia, harboring $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ (Sola et al., 2022), and in brown mussels (*Perna perna*) from Brazil, where $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{SHV-28}}$ were detected (Bueris et al., 2022). The oyster-derivate *K. pneumoniae* ST307 strain MUC-SAN exhibited a high mucoviscosity phenotype despite the absence of relevant virulence-associated genes. Recent studies have shown that mucoviscosity is not restricted to K1/K2 serotype isolates nor are it exclusively associated with the presence of the *rmp* locus (Fleeman et al., 2024), suggesting the existence of other genetic determinants of mucoviscosity in *Klebsiella pneumoniae*. Further studies are required to elucidate the molecular mechanisms underlying mucoviscosity in this strain. Additionally, these findings suggest that *K. pneumoniae* ST307 is associated with aquaculture products, likely influenced by fecal contamination from wastewater and agricultural runoff. *E. coli* ST38 and ST23 have emerged as significant human pathogens, often implicated in urinary tract and bloodstream infections (Pitout et al., 2019). Moreover, these sequence types have been detected in animals, wastewater, and food sources. Convergence of resistance and ability to harbor virulence-associated genes typical of both extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (IPEC) is the concerning (Roy Chowdhury et al., 2023). In our study, the high virulence potential of *E. coli* CRO1-OMV (ST38) and FLOR1-OMV (ST23) strains in the *G. mellonella* infection model confirms their pathogenicity, suggesting that oysters may harbor WHO critical priority pathogens with significant health risks, especially for susceptible populations.

Metal tolerance operons, such as the *ars* operon, play a crucial role in how pathogenic bacteria survive in hostile environments, including within a host. The *ars* operon provides bacteria with a mechanism to detoxify arsenic, reducing arsenate to arsenite and pumping it out of the cell (Ben Fekih et al., 2018). Then bacteria can survive in arsenic-rich environments, including in tissues and organs where arsenic could accumulate such as liver, kidneys, and the urinary tract of hosts (Lawal et al., 2021). On the other hand, molecular mechanisms involved in bacterial arsenic tolerance can sometimes confer cross-protection against other stresses, such as oxidative stress or antimicrobial agents (Mateos et al., 2017). Moreover, the presence of *ars* tolerance operons can influence the expression of other genes related to virulence. For

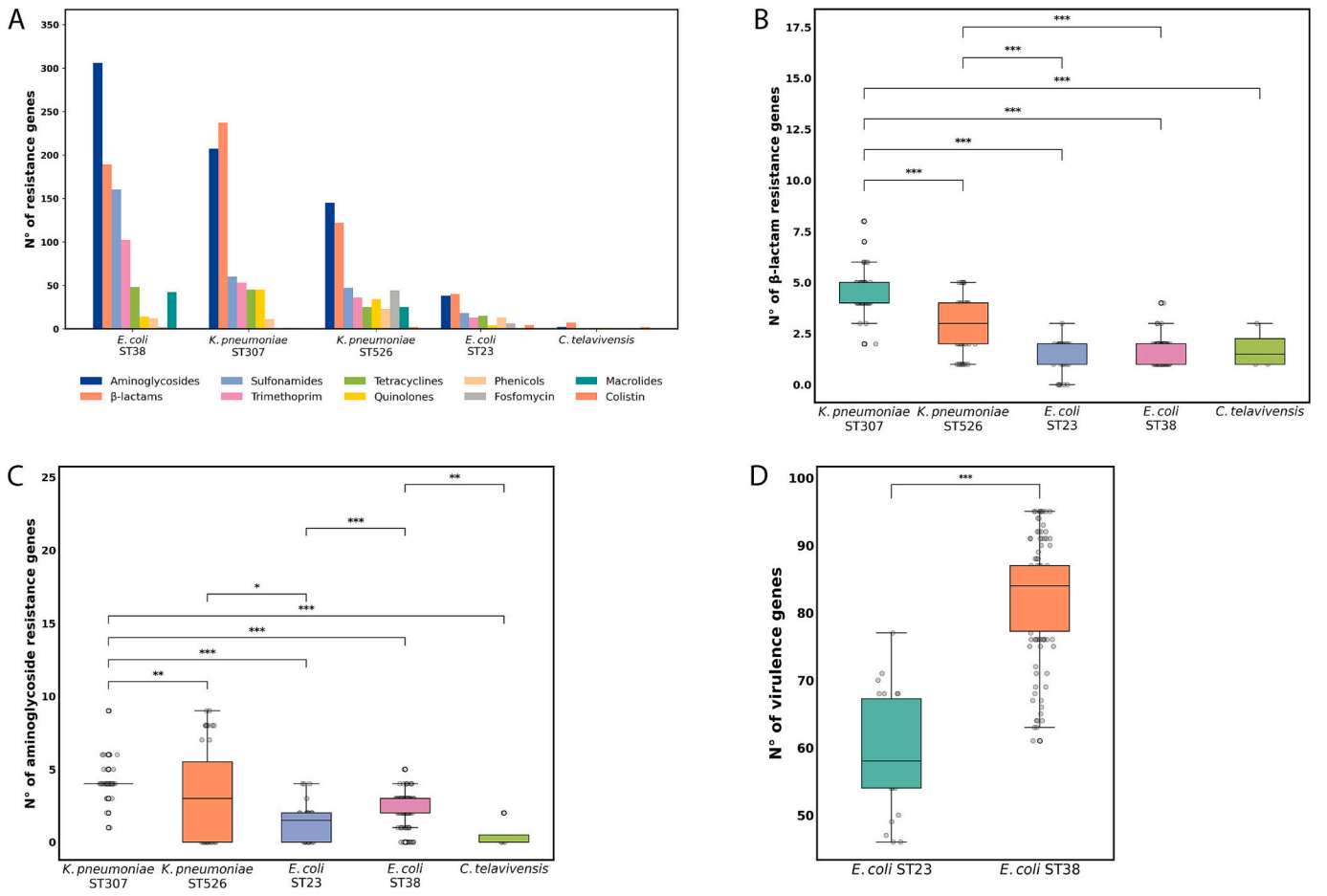

Fig. 6. Distribution and comparison of antimicrobial resistance and virulence genes across *Klebsiella pneumoniae* ST307 ($n = 47$) (South America), *Escherichia coli* ST23 ($n = 28$) (South America), *E. coli* ST38 ($n = 122$) (South America), *K. quasipneumoniae* ST526 ($n = 44$) (global), and *Citrobacter telavivensis* ($n = 4$) (global). (A) Bar plot showing the total number of resistance genes by sequence type. (B) Boxplot comparing the number of β-lactamase genes per strain among *K. pneumoniae* ST307, *E. coli* ST23, *E. coli* ST38, *K. quasipneumoniae* ST526, and *C. telavivensis*. (C) Boxplot comparing the number of aminoglycoside resistance genes per strain across the same sequence types. (D) Boxplot comparing the number of virulence genes between *E. coli* ST23 and ST38 from South America. Boxes represent the median and interquartile range (IQR), whiskers extend to 1.5× IQR, and dots beyond the whiskers represent potential outliers. Pairwise comparisons were performed using the Mann-Whitney *U* test, with significance levels indicated as ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

example, by maintaining cellular homeostasis under metal stress, bacteria can allocate more resources toward producing adhesion factors. In some bacterial species it has been demonstrated that arsenic tolerance may increase siderophore and extracellular polysaccharide production indirectly boosting pathogenic traits (Song et al., 2025). In the same way, arsenic tolerance can induce overexpression of multidrug resistance efflux proteins (Song et al., 20255). Resistance to arsenic and cadmium have been linked to human infection by *Staphylococcus saprophyticus* suggesting that resistance to these metals is relevant for pathogenicity (Lawal et al., 2021). In Gram-negative, carbapenem-resistant *K. pneumoniae* ST258 has emerged as an important source of hospital death. A previous study, it has suggested that the ars gene cluster, containing arsH, appears to have aided survival in natural killer cells (Hao et al., 2017; Ben Fekih et al., 2018). Survival in amoeba has been enhanced by the presence of an arsRBC operon in *E. coli* (Hao et al., 2017). In brief, *ars* tolerance operons in *E. coli* ST23 and ST38 could enhance bacterial resilience against host defenses and environmental stresses, thereby indirectly contributing to their pathogenic potential, since they act as survival tools that enable pathogens to thrive in challenging conditions, influencing their ability to cause disease.

The identification of *C. telavivensis* (strain CIT-FLOR) is other epidemiologically relevant results, since this novel species has not been identified outside clinical settings, so far. Moreover, this study demonstrates that this species has acquired clinically relevant antibiotic-resistant genes (*i.e*, $bla_{\text{CTX-M-15}}$) becoming a potential novel critical priority pathogen, which should be monitored in clinical settings. On the other hand, the identification of human-associated *K. quasipneumoniae* (strain KQ-FLOR) of ST526, producing the SHV-5 ESBL in oysters, in this study, confirm adaptation of this emergent clone at the human-food interface.

Brazil's National Program for Hygienic-Sanitary Control of Bivalve Mollusks (Brazil's National Program for Hygienic-Sanitary Control of Bivalve Mollusks (PNCMB), 2012) regulates shellfish safety by classifying harvesting areas based on *E. coli* levels: unrestricted (<230 MPN/100 g), conditionally approved (230–46,000 MPN/100 g), and closed (≥46,000 MPN/100 g). Mollusks from conditionally approved areas require depuration, heat treatment, or viscera removal before commercialization. While this system mitigates microbial risks, its reliance on *E. coli* as an indicator fails to account for other critical pathogens, particularly antimicrobial-resistant bacteria. The detection of WHO-critical priority pathogens in oysters highlights the need to integrate molecular surveillance of these bacteria into the PNCMB, enhancing food safety measures and public health protection.

Noteworthy, these critical priority pathogens isolated from RTE raw oysters carried the *ars* operon, as well as other heavy metal operons, and a global in silico analysis of all publicly available genomes confirmed that in South American clones of *E. coli* ST23 and ST38, and *K. pneumoniae* ST307, the *As* operon seem to be intrinsic, which could

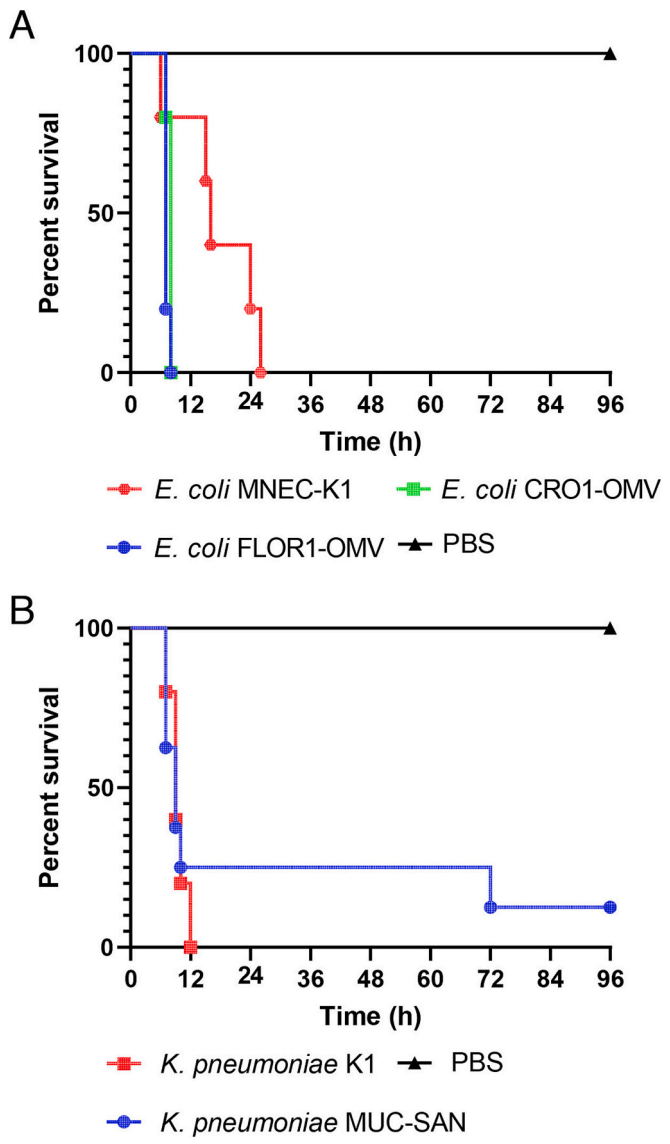

Fig. 7. Survival curves of *Galleria mellonella* larvae following infection with bacterial strains. Larvae were infected with 10^5 CFU/larva of *E. coli* strains CRO1-OMV and FLOR1-OMV, and 10^6 CFU/larva of *K. pneumoniae* strain MUC-SAN. Strains CRO1-OMV and FLOR1-OMV resulted in 100 % mortality of *G. mellonella* larvae within 9 h post-infection, whereas the virulent control *E. coli* strain MNEC-K1 induced 100 % mortality by 25 h post-infection. In contrast, strain MUC-SAN caused 85 % mortality at 96 h post-infection, while the virulent control *K. pneumoniae* strain K1 achieved 100 % mortality within 12 h post-infection. The uninfected control group (black line) was treated with 10 µL of PBS and showed no mortality. Each experiment was conducted with groups of 10 larvae per strain, with two technical replicates performed to ensure reproducibility.

favored the persistence and adaptation of these antimicrobial-resistant clones to impacted environments, colonizing related ecosystems and their fauna and flora, which could be part of the food chain. Interestingly, heavy metal contaminants pose an environmental burden and exert selective pressures that promote the proliferation of AMR (Baker-Austin et al., 2006). Studies suggest that heavy metal-contaminated environments enhance the co-selection of ARGs and heavy metal resistance genes, particularly in multidrug-resistant Enterobacterales (Yang et al., 2018). Therefore, the co-selection of AMR genes and heavy metal resistance operons in Enterobacterales highlights the complex ecological interactions in polluted environments. The potential for gene transfer between environmental strains and human-associated pathogens raises the risk of amplifying resistance in the human microbiota, particularly in vulnerable populations exposed to contaminated seafood. Regarding As tolerance gene transfer, a limitation of this study was related to the genomic analysis by long read technology, which did not allow the assembly of all plasmids. Consequently, location and transfer of As tolerance genes were not possible to be determined. Moreover, although the incorporation of 16S rRNA sequencing or metagenomic analysis would provide a more comprehensive microbial profile, which has also been a limitation of our study, we have privileged a targeted investigation of critical priority pathogens following WHO global research priorities for antimicrobial resistance in human health (Bertagnolio et al., 2024). Another limitation of this study is the lack of identifying the sources of As pollution and antibiotic-resistant bacteria in related aquatic ecosystems, which directly impact the microbiological quality of seafood, including oysters. A study conducted in 2022 in Florianópolis, Brazil—one of the country's primary oyster farming regions—reported elevated levels of thermotolerant coliforms, reaching between 1100 and 2400 most probable number (MPN)/100 mL (Saldaña-Serrano et al., 2022). These high contamination levels are largely attributed to inefficiencies in wastewater treatment systems, a problem exacerbated during the summer due to increased tourist influx, which places additional strain on water treatment infrastructure. This issue is further compounded by the discharge of hospital wastewater containing critical human pathogens such as *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *E. coli*, *Enterobacter cloacae*, and *Staphylococcus epidermidis*, often released into sewage systems without adequate pretreatment (Dias et al., 2021). Consequently, these pathogens disseminate into river waters, posing risks to both public health and local aquatic fauna. Therefore, implementing stringent control measures in shellfish production areas is crucial, as the release of antibiotic-resistant pathogens presents significant threats to human and animal health. Furthermore, climate change may exacerbate these risks by increasing extreme weather events that compromise water treatment systems, favoring the proliferation of antibiotic-resistant bacteria and foodborne pathogens, ultimately threatening food security and public health (Furlan et al., 2024; Saleem et al., 2024).

Regarding As contamination, a study in Brazil indicated that coastal waters and marine sediments exhibit high As concentrations primarily due to anthropogenic activities such as emissions from zinc smelters, fertilizer plants, and mining operations for gold and iron. Additionally, natural processes, such as the accumulation of As within the porous structure of calcareous algae, contribute to elevated As levels in beach sand and coastal waters (Baeyens et al., 2019). We suggest that bivalve organisms including oysters may be periodically exposed to As due to human and natural factors in Brazil.

Finally, our findings underscore the urgent need for enhanced surveillance, stringent food safety measures, and effective management practices in aquaculture to mitigate the risks posed by AMR and environmental contaminants in raw oysters. Our results underscore the need for surveillance of clinically relevant antibiotic-resistant pathogens in RTE oysters, along with appropriate management strategies, whereas prioritizing monitoring efforts in anthropogenically polluted coastal areas with oyster farming operations are important to preventing contamination. Additionally, we advocate stricter regulations on oyster farming and sales, including more comprehensive testing for antimicrobial resistance, as well as the establishment of a traceability system to enhance regulatory oversight and ensure consumer safety. Collaborative efforts among researchers, regulatory agencies, and the aquaculture industry are essential to safeguard public health and the integrity of marine ecosystems. Ultimately, our study highlights the critical need for improved monitoring and regulation of seafood safety, particularly aquatic environments, to address the dual threat of antimicrobial resistance and environmental contamination.

5. Conclusion

This study underscores the significant public health risks associated with raw ready-to-eat oysters, particularly their role as reservoirs of antimicrobial resistance (AMR) and their involvement in potentially toxic metal bioaccumulation in Brazil. Elevated As concentrations in oysters highlight the need for more accurate quantification of inorganic As species to better assess potential risks to human health. The identification of ESBL-producing *Klebsiella pneumoniae* ST307, *Klebsiella quasipneumoniae* ST526, and *Escherichia coli* ST38 and ST23 emphasizes the role of seafood as vectors for clinically significant AMR pathogens. These findings suggest that shellfish, particularly oysters, represent a critical interface between environmental contamination and human health risks, further exacerbated by anthropogenic activities. Given the widespread consumption of oysters, these results highlight the urgent need for enhanced surveillance systems and stricter regulatory measures to mitigate the public health risks posed by contaminated seafood. Strengthening food safety frameworks and promoting sustainable aquaculture practices will be essential to protect public health and preserve the ecological roles of oysters. The detection of WHO-critical priority pathogens in oysters highlights the need to integrate molecular surveillance of these bacteria into the Brazilian National Program for Hygienic-Sanitary Control of Bivalve Mollusks (PNCMB), enhancing food safety measures and public health protection.

CRediT authorship contribution statement

Felipe Vásquez-Ponce: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nicolas Gamboa-Acuña:** Writing – review & editing, Software, Methodology, Formal analysis, Data curation. **Aline Pereira de Oliveira:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Johana Becerra:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis. **Jesus G.M. Pariona:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Leon de Oliveira Lima:** Writing – review & editing, Methodology, Data curation. **Aline de Carvalho Elias:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Maciel Santos Luz:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Mateus Rocha Ribas:** Writing – review & editing, Validation, Methodology, Formal analysis. **Gustavo Rocha:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Fernanda Esposito:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Patricio Muñoz:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Edson Gonçalves Moreira:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Cassiana Seimi Nomura:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Thais Sincero:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Edison Barbieri:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Nilton Lincopan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.116684.

Data availability

Data will be made available on request.

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