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Antimicrobial-resistant *Enterobacter cloacae* complex strains isolated from fresh vegetables intended for raw consumption and their farm environments in the Northwest of Spain

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ABSTRACT

Antimicrobial resistance is a global concern with significant public health implications. We investigated the role of fresh vegetables and their cultivation environments as reservoirs for antimicrobial-resistant *Enterobacter cloacae* complex (ECC) strains. The study focused on AmpC-producing ECC isolates and their resistance to colistin, a last resort antibiotic.

AmpC-producing ECC isolates were detected and confirmed in 10.2% of the 235 samples examined, with no significant difference ($p > 0.05$) in prevalence between farm and street market samples. Further analysis of 24 AmpC-ECC isolates revealed that 16.7% exhibited resistance to colistin.

A colistin-resistant *E. kobei* strain (AG07E) was detected in irrigation water from a vegetable farm for the first time in Spain. This strain carried the *mcr-9.1* gene, demonstrating transferability. It was included in ST56 which is predominantly reported in clinical *E. kobei* harbouring the *mcr-9* gene. Additionally, we identified a multidrug-resistant *E. kobei* strain (ZA03E) from carrot samples, exhibiting colistin resistance and potential human pathogenicity. This strain belonged to ST125 which has clonal relationships with strains in ST56.

Our findings emphasise the importance of monitoring and addressing antimicrobial-resistant ECC strains in fresh vegetables and their production environments, particularly the water, to mitigate potential risks to public health from a One Health perspective.

1. Introduction

Antimicrobial resistance (AMR) is a growing global public concern as it can result in severe infections and high mortality rates. The transmission and dissemination of AMR among various reservoirs, including humans, animals, plants, and the environment, necessitate a comprehensive "One Health" approach to effectively combat this issue. This perspective is echoed by prominent international organizations in a recent publication (World Health Organization et al., 2023). In that specific report, the pivotal role of the agricultural environment, including the irrigation water and other agricultural factors, in facilitating the transmission of antimicrobial-resistant bacteria to crops was recognized as a critical area requiring further investigation. Research in this area is supported by the supported by the EFSA BIOHAZ Panel (Koutsoumanis et al., 2021), which emphasised the role of food-producing environments, including vegetable production, in the dissemination of AMR throughout the food system.

It is generally acknowledged among health institutions worldwide that incorporating vegetables and fruits into our daily diet is essential for promoting good health and reducing the risk of diseases. However, it must be noticed that this food category is not devoid of potential hazards to human health. From a microbiological standpoint, a recent report by EFSA and ECDC (2022) showed an increasing trend in the number of cases and outbreaks associated with vegetables and plant-based products. These microbial concerns might increase due to the potential of vegetables to serve as a source of antimicrobial-resistant bacteria, among which resistant Enterobacteriaceae can be present (Blaak et al., 2014; Chelaghma et al., 2021; Manaia, 2017). The role of fresh produce

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as a reservoir of bacteria displaying AMR is not as well-established as in foods of animal origin, which are subjected to regular monitoring by authorities (EFSA & CDC, 2023). Nevertheless, studies conducted over the last decade have increasingly identified fresh vegetables as a potential reservoir of ESBL/AmpC-producing Enterobacteriaceae in various countries, such as, the Netherlands (Blaak et al., 2014), Italy (Iseppi et al., 2018), South Africa (Richter et al., 2019), Spain (Pintor-Cora et al., 2021), and the USA (Moon et al., 2022). However, information regarding the detection and characterization of members of the *Enterobacter cloacae* complex (ECC) exhibiting these resistance determinants, either alone or in combination with other resistance mechanisms, in fresh produce remains limited. ECC has been recognized as one of the most common Enterobacteriaceae groups leading to nosocomial infections due to the general spread of some β-lactamases types (Davin-Regli et al., 2019). While this bacterial complex is relatively uncommon in foodborne illnesses compared to other bacterial pathogens, the potential presence of antimicrobial-resistant strains of these bacteria in the food chain, as well as the potential dissemination or transmission of their AMR determinants, could pose a heightened risk to public health. On the other hand, the emergence of multidrug resistance (MDR), including resistance to last-resort antibiotics, such as colistin, has increased the interest in the ECC (Annavajhala et al., 2019).

The rise in prevalence of colistin-resistant *Enterobacterales* has been attributed to the transmission of resistant isolates to humans from various sources, including animals, food and due to clinical treatments, as elucidated in the study by Binsker et al. (2022). Colistin resistance in Enterobacteriaceae is often associated with the presence of a mobile variant of the *mcr* gene (Zurfuh et al., 2016). The identification of *mcr*-positive Enterobacteriaceae in vegetables remains relatively rare, with only a limited number of documented instances reported in previous studies (Chelaghma et al., 2022; Liu et al., 2019; Manageiro et al., 2020; Zurfuh et al., 2016; Luo et al., in 2017). The detection of variants of the *mcr* gene in ECC strains isolated from vegetables is even rarer (Moon et al., 2022).

Due to the increasing importance of the food chain as way of dissemination of AMR bacteria and the potential impact on human health, this study aimed to determine whether fresh vegetables, and their growing environments, serve as reservoirs of antimicrobial-resistant *Enterobacter cloacae* complex strains and to characterise ECC isolates in terms of their production of AmpC β-lactamases, as well as other clinically significant β-lactamases (ESBLs/carbapenemases) and resistance to colistin.

2. Material and methods

2.1. Bacterial isolation and identification

A total of 145 samples of fresh vegetables were collected from Spanish farms and street markets. Vegetable samples included lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), carrot (*Daucus carota* subsp. *sativus*), escarole (*Cichorium endivia* var. *latifolium*), pepper (*Capsicum annuum*), parsley (*Petroselinum crispum*) and coriander (*Coriandrum sativum*). Ninety additional samples from farm environments were also studied (soil, irrigation water, air, and worker hands).

Ten grams of each vegetable and soil sample were homogenised with 90 ml of buffered peptone water (BPW; Oxoid, Thermofisher, UK). Foreign matter and nonedible parts were removed using sterile tools but no further cleaning steps were conducted. Water samples were processed by filtering 100 ml of the sample through a 0.45 μm filter, and the filter was then soaked in 100 ml of BPW. Air samples (100 l) were collected in farms using a microbial air sampler (Biotest Hycon, Dreieich, Germany) equipped with a ChromAgar Enterobacteria plate (ChromAgar, Paris, France). One hand swab sample was taken from each farm worker, and the swab was then placed in a 10 ml tube containing BPW.

Homogenates were incubated for 24 h at 37 °C. One loopful of the enriched solution was streaked onto different chromogenic media: ChromAgar ESBL (ChromAgar), Chromagar KPC (ChromAgar) and Agar Mconkey (Oxoid) supplemented with 16 μg/ml cefoxitin sodium salt (Sigma-Aldrich, Merck, Germany) for the detection of suspected β-lactam resistant isolates. Plates were incubated at 37 °C for 24 h and colonies with morphology associated with β-lactam resistance in accordance with manufacturer's instructions were selected for additional characterization.

Isolates were primarily identified as belonging to the *Enterobacter cloacae* complex by matrix-assisted laser desorption/ionisation - time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik, Germany).

2.2. Antimicrobial susceptibility testing

B-Lactamase production in the suspected isolates was confirmed using the MAST D72C AmpC and ESBL detection kit (MAST group, UK), based on the disk diffusion technique, which classified the isolates as ESBL producer, AmpC inducible producer, AmpC non-inducible producer, ESBL and AmpC co-producer or suspected carbapenemase producer. Suspected carbapenemase producers were confirmed using the MAST-D73C kit (MAST group).

Detection of multidrug resistant isolates was carried out also by the disk diffusion method using a selection of antimicrobial agents of different categories which included ampicillin, cefuroxime, cefotaxime, cefepime, aztreonam, imipenem, gentamicin, ciprofloxacin, trimethoprim-sulphamethoxazole and chloramphenicol. Results were interpreted conforming to the EUCAST and CLSI breakpoint values (CLSI, 2023; EUCAST, 2023). Colistin resistance was detected among the isolates by agar dilution methodology in Mueller Hinton plates (Oxoid) supplemented with 2 μg/ml of colistin (ADATAB, MAST group, UK) adjusting the bacterial inoculum to 10^4 CFU per spot. Suspected colistin-resistant colonies were confirmed by a microdilution method.

The minimum inhibitory concentration (MIC) was determined by broth microdilution following the guidelines of ISO 20776–1:2021 (ISO-International Organization for Standardrization, 2021) and interpreted conforming to the breakpoints provided by EUCAST (2023). MIC for colistin, cefotaxime and ceftazidime were determined in those isolates which showed a resistant phenotype to these antimicrobial agents in the previous testing. The studied antibiotic concentrations ranked from 0, 25–256 μg/ml. *E. coli* ATCC 25922 was used as a negative control strain.

2.3. Genetic relationships among the ECC isolates

Multilocus sequence typing (MLST) procedure was carried out using the scheme designed by Miyoshi-Akiyama et al. (2013) for isolates belonging to the *Enterobacter cloacae* complex, which included 7 genes: *dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB* and *rpoB*. Each gene was amplified independently by PCR, purified (GFX PCR DNA and Gel Band Purification Kit, Cytiva, USA) and sequenced by Sanger technology. The resulting sequences were aligned against a reference sequence and trimmed to target size using clustalW in MEGA 11 software (Tamura et al., 2021). Alleles and sequences types (ST) were obtained from PUBMLST database (Jolley et al., 2018).

Phylogenetic analysis was conducted by aligning and applying the neighbor joining algorithm to a concatenation of the seven housekeeping genes of the strains, with the distances estimated by the maximum composite likelihood model and a bootstrapping of 1000 replications using MEGA11 software (Tamura et al., 2021). The reference genomes available on REFSEQ for the species such as *E. cloacae*, *E. mori*, *E. ludwigii*, *E. kobei*, *E. asburiae*, *E. cancerogenus* and *E hormaechei* were used for identification purpose and assessing clonal relationships.

Pulsed-field gel electrophoresis (PFGE) analysis was conducted using a standardised protocol by PulseNet (https://www.cdc.gov/pulsenet/index.html). The restriction enzyme *Xba*I (New England Biolabs, Ipswich, MA) and a CHEF-DR III apparatus (Bio-Rad) were employed

following the procedure described by Pablos et al. (2010).

2.4. Genetic characterization by whole genome sequencing (WGS)

Genomic DNA of selected isolates was extracted using Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, US) and quantified using Qubit fluorometer (Thermo Fisher Scientific, US). Bacterial genomes were sequenced using a Novaseq sequencer (Illumina, San Diego, CA) and/or a GridION device (Oxford Nanopore Technologies, UK). Assembly was done using the PATRIC web platform (Wattam et al., 2017) or Flye (Kolmogorov et al., 2019), respectively.

Assembled contigs were annotated using the RAST online platform (https://rast.nmpdr.org/rast.cgi). CARD was used for the detection of antimicrobial resistance genes and BLDB (http://bldb.eu/) for detection and characterization of β-lactamases. SnapGene Viewer (www.snapgene.com) was used for manual analysis and detection of the recombination hotspot described by Paauw et al. (2010) on adherent isolates, checking each gene contained in this region through BLAST NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Substitutions on PhoP/Q, PmrA/B and MgrB regulation proteins were assessed on colistin-resistant isolates by comparison against sequences obtained from reference genomes of *E. ludwigii* (GCF_001750725.1), *E. kobei* (GCF_000534275.1), *E. hormaechei* (GCF_019048625.1) and *E. asburiae* (GCF_007035805.1) using ClustalW in Mega 11 software (Tamura et al., 2021).

2.5. Pathogenic potential of selected isolates

The pathogenic potential of selected representative isolates was tested in a cell infection assay, using the HT29 human colon cell line (ATCC® HTB-38TM) and primary mouse embryonic fibroblasts (MEF). HT-29 cells were cultured in McCoy's 5a medium (Gibco) and MEF were maintained and cultured in Dulbecco's Modified Eagle Medium (DMEM), both of which were supplemented with 10% of heat-inactivated foetal bovine serum (FBS, Invitrogen). Cells were grown in round glass coverslips placed in 24 tissue-culture plates and then inoculated with an adjusted overnight culture (5×10^9 CFU/ml) of each strain so as the multiplicity of infection (MOI) was 100 bacteria per each cell. Infected cell plates were centrifuged for 4 min at 200×*g* in order to synchronise infections and then incubated at 37 °C with 5% CO_2 for 3 h. *Escherichia coli* DH5-α at MOI 200:1 was used as a non-pathogenic control strain.

After incubation, cells were washed four times and fixed with a cold 3.2% paraformaldehyde solution and incubated at room temperature for 20 min. Cells were then treated with Triton X-100 (0.1%) for 5 min at room temperature in order to permeabilize the membranes and washed five times with PBS. Fluorescent molecules used to dye the preparation were Atto-594 phalloidin (Sigma), which binds polymerised F-actin; fluoroshield containing DAPI (Sigma Aldrich) to stain DNA and *Enterobacter cloacae*-specific polyclonal antibodies which were later bound to a secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). All preparations were examined with a Nikon A1R confocal scanning laser microscope equipped with 403 nm and 561 nm lasers. Images were captured at random with a × 40 Plan-Fluor 1.3 NA objective, and processed using the NIS-Elements 3.2 software.

2.6. Conjugation assays

Resistance gene transfer assays were performed using *E. coli* CECT 670 as the recipient. Exponential cultures (*ca.* 4 h) of donor and recipient strains were centrifuged and resuspended in 100 μl of TSB in a 2:1 donor-recipient ratio. This mixture was inoculated onto Columbia blood agar plates (Oxoid) and incubated at 37 °C for 48 h. Bacterial biomass was then recovered, resuspended in saline solution (0.85% NaCl) and appropriate dilutions were inoculated into TSA supplemented with 32 mg/l of streptomycin and of 2 mg/l colistin for transconjugant selection. Suspected transconjugants were verified by PCR (Gorecki et al., 2022) and MALDI-TOF identification.

2.7. Statistical analysis

Basic descriptive statistics were calculated using an Excel datasheet. A chi-square test was used to determine the significant association between sample origin (street markets; farms) and AmpC- sample (positive; negative). The IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA) program was used for this purpose.

3. Results

3.1. Isolation and identification of bacterial strains

Twenty-four bacterial strains were isolated from diverse sources, including tomato (5), carrot (4), lettuce (3), cucumber (3), irrigation water (3), parsley (2), escarole (1), coriander (1), farm handler (1), and soil (1). Multi-locus typing analysis (MLSA) revealed that the isolates belonged to the ECC, with 10 identified as *E. ludwigii*, 8 as *E. hormaechei*, 3 as *E. mori*, 2 as *E. kobei*, and 1 as *E. asburiae* (Table 1). It must be noted that only 6 out of the 24 isolates showed identical results with MALDI-ToF and MLSA identification (Table 1).

AmpC-producing ECC isolates were detected and confirmed in 10.2% out of 235 samples examined, 17 out of 180 farm samples (9.4%) and 7

Table 1
Distribution of 24 antimicrobial-resistant isolates of *E. cloacae* complex from fresh produce and their farm environments (n = 235).

Isolate no.	MS MALDI-TOF Identification	MLSA (7 loci)	Source	Sample origin	AMR phenotype[a]
AG07E	*E. cloacae*	*E. kobei*	Irrigation water	Farm A	iAmpC
AG32E	*E. ludwigii*	*E. ludwigii*	Irrigation water	Farm B	AmpC + porin loss
AG38E	*E. cloacae*	*E. hormaechei*	Irrigation water	Farm B	cAmpC
CI24E	*E. ludwigii*	*E. ludwigii*	Coriander	Farm B	cAmpC
ES13E	*E. ludwigii*	*E. ludwigii*	Escarole	Farm B	iAmpC
LE07E	*E. cloacae*	*E. ludwigii*	Lettuce	Street market1	iAmpC
LE43E	*E. cloacae*	*E. hormaechei*	Lettuce	Farm C	iAmpC
LE47E	*E. cloacae*	*E. hormaechei*	Lettuce	Farm C	cAmpC
MA17E	*E. cloacae*	*E. mori*	Farm handler	Farm B	iAmpC
PE22E	*E. cloacae*	*E. mori*	Cucumber	Farm B	iAmpC
PE23E	*E. cloacae*	*E. ludwigii*	Cucumber	Farm B	iAmpC
PE24E	*E. cloacae*	*E. mori*	Cucumber	Farm C	iAmpC
PJ29E	*E. cloacae*	*E. ludwigii*	Parsley	Street market3	iAmpC
PJ39E	*E. ludwigii*	*E. ludwigii*	Parsley	Farm B	cAmpC
SU38E	*E. cloacae*	*E. ludwigii*	Soil	Farm B	iAmpC
TO01E	*E. cloacae*	*E. hormaechei*	Tomato	Street market2	cAmpC
TO04E	*E. cloacae*	*E. hormaechei*	Tomato	Street market2	iAmpC
TO39E	*E. cloacae*	*E. hormaechei*	Tomato	Farm C	cAmpC
TO40E	*E. cloacae*	*E. hormaechei*	Tomato	Farm C	iAmpC
TO42E	*E. cloacae*	*E. hormaechei*	Tomato	Farm C	AmpC + ESBL
ZA02E	*E. cloacae*	*E. ludwigii*	Carrot	Farm B	iAmpC
ZA03E	*E. kobei*	*E. kobei*	Carrot	Street market2	iAmpC
ZA15E	*E. asburiae*	*E. asburiae*	Carrot	Street market2	iAmpC
ZA28E	*E. cloacae*	*E. ludwigii*	Carrot	Street market3	iAmpC

[a] AmpC phenotypes (MASTDISCS Combi -AmpC, ESBL & Carbapenemase detection set D72C): inductive (iAmpC), constitutive (cAmpC), co-producing AmpC and ESBL enzymes (AmpC + ESBL); AmpC + porin loss, confirmed by MASTDISCS Combi Carba plus (D73C).

out of 55 street market samples (12.7%). The difference in prevalence between farm samples and street market samples was not statistically significant ($p > 0.05$).

Isolates were mostly collected from MacConkey agar supplemented with cefoxitin (FOX) 16 mg/ml (21 isolates, 87,5%), being 3 isolates recovered on ESBL ChromAgar. The AmpC ESBL Carbapenemase Detection Set (D72C) confirmed the 24 ECC strains as AmpC producers. Among them, 8 were corresponding with a constitutive phenotype (5 *E. hormaechei* and 3 *E. ludwigii*; Table 1). They were found on tomato (3), parsley (1), coriander (1), lettuce (1) and irrigation water (2). One isolate from tomato (TO42E) was also an ESBL-coproducer and the isolate AG32E, from irrigation water, hydrolysed carbapenems besides showing an inducible AmpC profile.

3.2. Phenotypic resistance of vegetable ECC-isolates

Table 2 shows the antimicrobial susceptibility patterns of the 24 ECC isolates obtained from vegetables and farm environments. All isolates exhibited resistance to cefoxitin. The addition of clavulanic acid did not enhance sensibility to amoxicillin, reflecting the wild-phenotype of this enterobacterial group. Notably, over half of these isolates demonstrated resistance to cefuroxime (CXM), while 25% showed resistance to piperacillin (PIP). Additionally, resistant rates of 20.8% were observed for gentamicin (GEN) and cefotaxime (CTX), 16.7% for ticarcillin (TIC), ceftazidime (CAZ), aztreonam (ATM), and colistin (CL) respectively. Among the eight isolates showing a constitutive AmpC phenotype, TO01E isolated from tomato and LE47E isolated from lettuce exhibited enhanced resistance (MIC value $\geq$ 256 mg/l) to CTX and CAZ, along with the absence of sensibility to the fourth-generation cephalosporin (4 GC) cefepime (Table 1).

The *E. ludwigii* (LE07E) and *E. kobei* (ZA03E) isolates demonstrated resistance to two different antimicrobial categories, in addition to the β-lactams group, indicating a multidrug-resistance (MDR) phenotype (8,3%). These two strains, isolated from lettuce and carrot, along with two others from escarole (ES13E) and irrigation water (AG07E), exhibited resistance to colistin on specific agar plates (16.7%), which were further confirmed by their MIC values.

3.3. Genetic features and pathogenic potential of antimicrobial-resistant isolates

Table 3 shows the genetic characteristics of a selection of 11 ECC isolates. Bacterial genomes presented and analysed in this study are available at NCBI genbank (https://www.ncbi.nlm.nih.gov/genbank/) under Bioproject IDs PRJNA998770. These isolates harboured genetic determinants for both the FosA2 enzyme, conferring resistance to fosfomycin, and the OqxA protein, which is a multidrug efflux pump. A diversity of ACT variants was detected through the WGS analysis. ACT-54 (strains AG32E, LE07E and LE43E) and ACT-47 (strains AG38E, LE47E and TO01E) were the most prevalent variants. Interestingly, these variants were detected in strains isolated from water and vegetables. Two *E. kobei* strains (AG07E and ZA03E) and two *E. ludwigii* strain (LE07E and ES13E) were found to carry the pmrHFIJKLM operon, as well as the *pmrE (ugd)* and *pmrC* (*eptA*) genes, which are potential determinants of polymyxin resistance. Analysis of aminoacidic substitutions in regulation genes against the reference sequence revealed that both AG07E and ZA03E carried N233T and Q168P substitutions in PmrB, and ZA03E also carried A72T substitution in pmrA.

No *mcr* genes were detected in three out of the four strains that exhibited phenotypic resistance to colistin. Nevertheless, it is noteworthy that *E. kobei* AG07E was found to harbour the *mcr-9.1* gene, indicating the presence of a specific mechanism conferring resistance.

To assess the pathogenicity of the isolates, 15 strains were selected and studied. Interestingly, none of them exhibited cytotoxic effects on four different mammalian cell lines, including human HT-29 colon cells and mouse fibroblasts. However, 3 vegetable isolates (ZA03E, TO42E, and LE47E) demonstrated the ability to adhere to HT-29 human colon epithelial cells, suggesting potential adhesive properties (Supplementary Fig. 1).

The analysis of the recombination hotspot in *E. kobei* ZA03E

Table 2
Antimicrobial susceptibility profiles of 24 vegetable ECC isolates.

Isolate no.	Tested antimicrobial [a]																
	AMC	AMP	TIC	PIP	CXM	FOX	CTX	CAZ	FEP	ATM	IPM	GEN	CIP	SXT	CHL	TET	CL
AG07E	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	R (128)[b]
AG32E	R	R	S	S	R	R	S	S	S	S	I	S	S	S	S	S	S
AG38E	R	R	S	I	R	R	I (2)	S	S	S	S	S	S	S	S	S	S
CI24E	R	R	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S
ES13E	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	R (4)
LE07E	R	R	S	R	R	R	R (4)	S	S	R	S	S	S	S	R	S	R (4)
LE43E	R	R	S	S	R	R	R (4)	R (4)	S	S	I	S	S	S	S	S	S
LE47E	R	R	R	R	R	R	R (>256)	R (256)	I	R	S	S	S	S	S	S	S
MA17E	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PE22E	R	S	S	S	S	R	S	S	S	S	S	S	I	S	S	S	S
PE23E	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PE24E	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
PJ29E	R	R	I	R	R	R	S	S	S	S	S	R	S	S	S	S	S
PJ39E	R	R	S	S	S	R	S	S	R	S	S	S	S	S	S	S	S
SU38E	R	R	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S
TO01E	R	R	R	R	R	R	R (>256)	R (256)	R	R	S	S	S	S	S	S	S
TO04E	R	R	S	S	R	R	I (2)	I (4)	S	S	S	R	S	S	S	S	S
TO39E	R	R	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S
TO40E	R	R	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S
TO42E	R	R	R	R	R	R	R (>256)	R (128)	I	R	S	S	S	S	S	S	S
ZA02E	R	R	S	S	R	R	S	S	S	I	S	R	S	S	S	S	S
ZA03E	R	R	I	S	R	R	S	S	S	S	S	R	S	S	S	S	R (64)
ZA15E	R	R	S	S	S	R	S	S	S	I	S	R	S	S	S	S	S
ZA28E	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S
% resistance	100.0	91.7	16.7	25.0	62.5	100.0	20.8	16.7	8.3	16.7	0.0	20.8	0.0	0.0	4.2	0.0	16.7

[a] Eucast cutoffS 2021 (https://eucast.org/; accessed on September 29, 2021) except from Tetracycline (CLSI, 2023, p. 33rd Ed.). AMC, Amoxicillin-clavulanate; AMP, Ampicillin; TIC, Ticarcillin; PIP, Piperacillin; CXM, Cefuroxime; FOX, Cefoxitin; CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; ATM, Aztreonam; IPM, Imipenem; GEN, Gentamicin; CIP, Ciprofloxacin; SXT, Trimethoprim-Sulfamethoxazole; CHL, Chloramphenicol; TET, Tetracycline; CL, Colistin.

[b] MIC breakpoints (mg/L) displayed in brackets.

Table 3
Genetic features from whole genome sequences of vegetable-ECC isolates.

	ACT variant[a]	AMR-related genes						Aminoacidic substitutions: polymyxin-resistance regulation proteins				
		fosA2	*oqxA*	*PmrE* (ugd)	*PmrC* (eptA)	*pmrHFIJKLM* operon	*mcr-9.1*	pmrA	pmrB	phoP	phoQ	mgrB
AG07E	ACT-9 (100%)	+	+	+	+	+	+	–	Q168P, N233T	–	–	–
AG32E	ACT-54 (99,7%)	+	+	+	+	+	–	–	–	–	–	–
AG38E	ACT-197 or ACT-47 (99,4%)	+	+	+	+	–	–	A31T, M126L	V94A, Q125L, A131D, Y325N, A332D, A333E	–	R137L, G483V	–
ES13E	ACT-109 (100%)	+	+	+	+	+	–	–	–	–	K2R	–
LE07E	ACT-54 (100%)	+	+	+	+	+	–	–	–	–	E242D, K377Q	–
LE43E	ACT-54 (99,4%)	+	+	+	+	–	–	M126L	Q125L, I280T	–	–	–
LE47E	ACT-47 (99,7%)	+	+	+	+	–	–	M126L	V94A, Q125L, A131D, K222R, A233P, R276Q, I280T, A333E	–	G483V	–
TO01E	ACT-47 (99,4%)	+	+	+	+	–	–	M126L	V94A, Q125L, A131D, K222R, A233P, Y325H	–	G483V	–
TO42E	ACT-14 (100%)	+	+	+	+	–	–	–	L76F, Q125L, A131D, Y325N, A333E	–	–	–
ZA03E	ACT-28 (100%)	+	+	+	+	+	–	A72T	Q168P, N233T	–	–	–
ZA15E	ACT-57 (100%)	+	+	+	+	+[b]	–	–	–	–	–	–

[a] ACT variant, percentage of aminoacidic identity against query sequence is shown in brackets.
[b] ZA15E isolate is lacking the *pmrM* gene in the *pmrHFIJKLM* operon.

indicated the presence of a 31 kb region (Fig. 1). This region is integrated next to a tRNA gene and carries a phage type integrase, with a different GC content (37%) compared to the genome (55%). Furthermore, the region contained 37 ORFs, including 21 hypothetical proteins. However, no genomic modules or islands were found in the other two adherent isolates (LE47E and TO42E).

3.4. Transfer of antimicrobial-resistance genes

Conjugation experiments were performed, demonstrating the successful transfer of the *mcr-9.1.* gene from *E. kobei* AG07E to the recipient strain. As a result, the recipient strain acquired resistance to colistin.

3.5. Molecular typing of the AmpC-ECC isolates from vegetables

Multilocus sequence typing (seven loci) identified a total of eight distinct sequence types (STs) (Fig. 2). In contrast, the allelic profiles of the strains AG32, MA17E, PE22E, PE24E, SU38E, and ZA02E did not correspond to any available STs in the pubMLST database for *E. cloacae*. Moreover, four novel alleles were found for *gyrB* and *leuS*, respectively, as well as two novel alleles for *pyrG* and one novel allele for *rplB* (Supplementary Table 1). On the other hand, the remaining strains exhibited allelic profiles that matched six out of seven loci with different STs.

*Xba*1-PFGE analysis revealed the presence of 21 distinct pulsetypes, with a minimum similarity coefficient of 90%. Three small clusters were identified, consisting of the vegetable strains PJ29E and ZA28E, TO40E and TO42E, and CL24E and PJ39E, respectively. One additional strain (ZA02E) was non-typeable (Supplementary Fig. 2).

4. Discussion

Overall, we confirmed AmpC-producing ECC in 10.2% of samples and significant differences among sample origins were not found. In contrast, other studies (Blaak et al., 2014; Moon et al., 2022; van Hoek et al., 2015) reported a much lower prevalence of AmpC-producing strains (ranging from 1,20%–2.27%) in various ready-to-eat vegetables. However, it is important to note that these studies focused on a broader bacterial range, mainly Enterobacteriaceae. Therefore, when comparing our results with the existing scientific literature, it becomes evident that fresh vegetables and their growing environments seem to be important reservoirs for AmpC-producing ECC.

Selective chromogenic agar isolation of colonies followed by Mass Spectrometry MALDI-ToF is a direct, general and fast method for species identification among the *E. cloacae* complex. However, only 37.5% of isolates matched the identification provided by sequence analysis of relevant representative loci. MLSA based on seven housekeeping genes has been used as a robust technique for identifying ECC isolates (Annavajhala et al., 2019). Most of *E. ludwigii* and *E. hormaechei* strains isolated in our work were associated with *E. cloacae* through MALDI-ToF. There is a compelling reason behind this discrepancy, as the

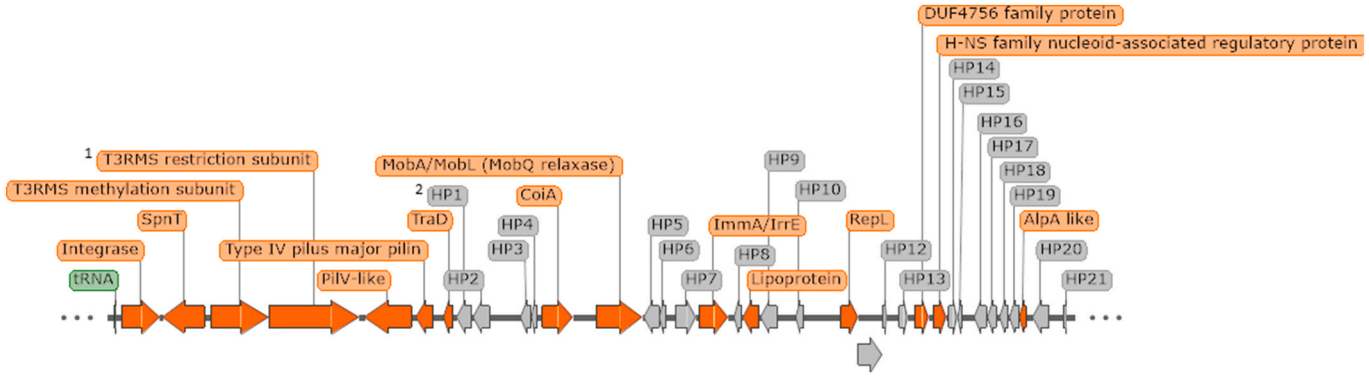

Fig. 1. Pathogenicity island from *E. kobei* ZA03E isolate. (1) T3RMS: type III restriction-modification system. (2) HP: hypothetical protein.

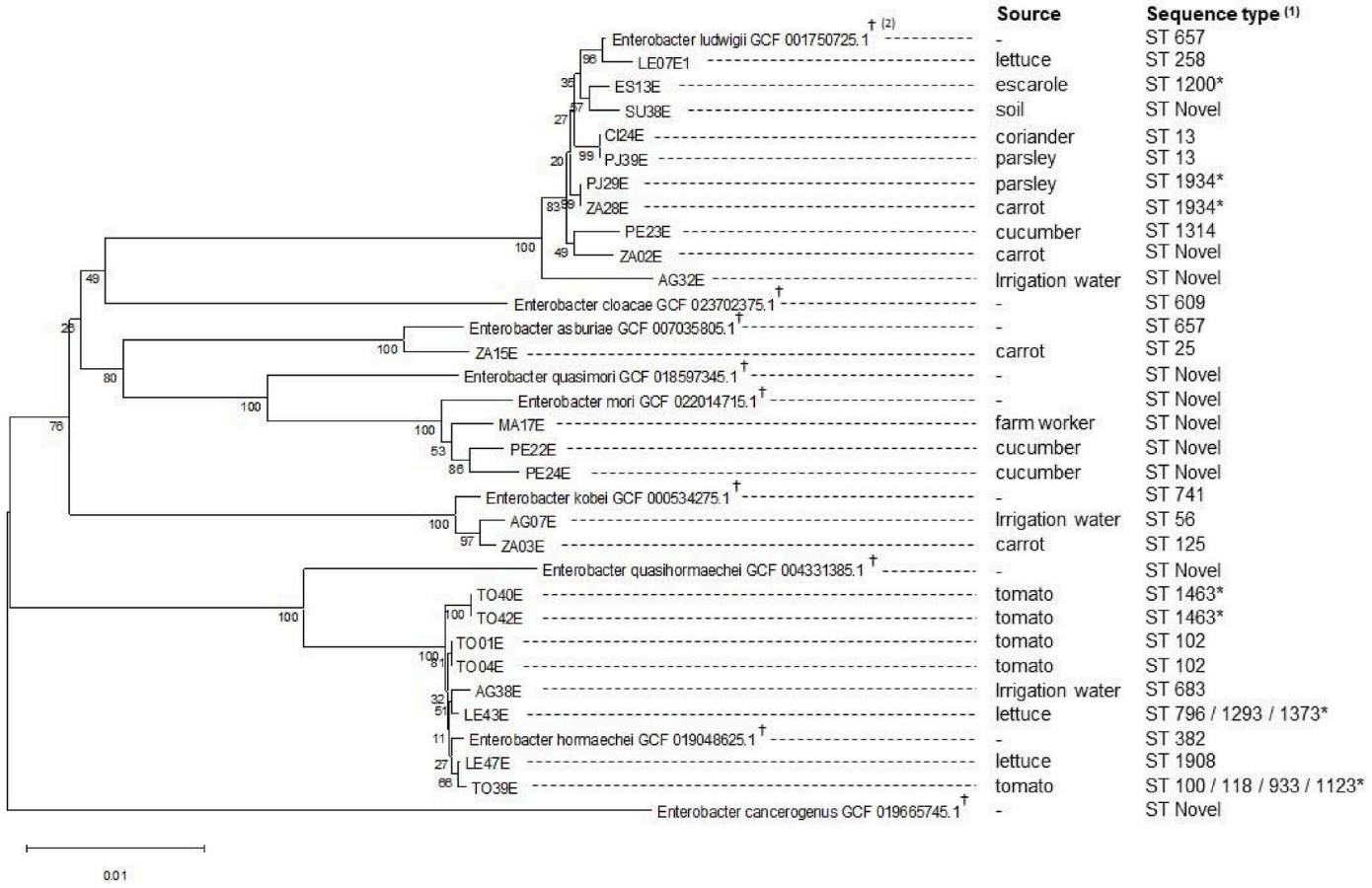

Fig. 2. Phylogenetic distance tree based on the multilocus sequence analysis (7 loci) of 24 antimicrobial-resistant ECC obtained from fresh produce and farm environments. Neighbor-joining method. Numbers at the nodes of the tree indicate bootstrap values obtained after 1000 replicates.
(1) ST, Sequence Type according to the *E. cloacae* database on pubmlst.org; symbols indicate 6/7 allelic coincidence (*) and a ST not described at pubMLST (−), respectively.
(2) †, the symbol indicates a reference sequence obtained from the Refseq database. Accession numbers for reference strains of *Enterobacter* spp. are provided.

database used (Bruker) primarily comprises *E. cloacae* strains within the ECC, whereas representation of *E. hormaechei* and *E. ludwigii* strains is limited to only one strain each. This underscores the necessity of expanding the database with a wider range of non-clinical strains from these specific bacterial species, aiming to enhance the accuracy of identification outcomes.

Among the 24 ECC isolates producing AmpC-betalactamases, two isolates from tomato and irrigation water were determined as potential co-producers, as they phenotypically exhibited other types of β-lactamases: ESBL or carbapenemase, respectively. For any member of Enterobacteriaceae with AmpC production, regardless if intrinsic chromosomal or plasmid encoded for AmpC expression, could be expected that other β-lactamases were produced by the same bacteria and becoming the phenotypic classification difficult to interpret (Tamma et al., 2019). This particular characteristic in those strains has clinical significance since it is frequently found that ECC isolates related to human disease are able to produce ESBL along with overexpressing AmpC (Zhou et al., 2018).

The antimicrobial susceptibility patterns of the studied ECC isolates (Table 2) reflected antimicrobial profiles compatible with the production of AmpC β-lactamases, which was also pointed out by the MAST D72C set (Table 1). Interestingly, we observed a great increase in MIC values for ceftazidime and cefotaxime (reaching 256 mg/l or higher) in two vegetable isolates of *E. hormaechei* (TO01E and LE47E). This finding suggests the presence of a constitutive AmpC phenotype with hyperproduction of the b-lactamase. Additionally, these isolates share the same ACT variant (ACT-47). Resistance to extended-spectrum cephalosporins, such as cefotaxime, ceftazidime, and aztreonam, have been reported in *Enterobacter* spp. from specific chromosomal mutation, usually in *ampD* gene that originally avoids high-level expression of the Beta-lactamase (Sanders & Sanders, 1997), or through plasmid-mediated antimicrobial resistance (Zhou et al., 2018).

The use of fourth-generation cephalosporins, such as cefepime, is noted more effective against AmpC-hyperproducing *Enterobacter* strains as reviewed by Davin-Regli et al. (2019). Moreover, isolates associated with the AmpC phenotype, without coproduction of other important β-lactamase enzymes, are considered to be completely controlled by the activity of cefepime and carbapenems (Tamma et al., 2019). Surprisingly, the strains TO01E and LE47E of *E. hormaechei*, which is an important emerging human pathogen, were not inhibited by cefepime. Therefore, this characteristic would confer clinical importance owing to the fact that it could cause both a direct public health concern by consumption of fresh vegetables, particularly to vulnerable populations, and, indirectly, by their contribution to a resistant-gene pool from which potentially pathogenic bacteria may acquire these genetic determinants. In this sense, the last reports by EFSA (2023) concluded that the presence in the food chain of enterobacteria harbouring AMR determinants, such as ESBL, AmpC, or showing both phenotypes, which can be encoded in plasmids with transferable capability, configured a relative high potential of risk for both occasional human pathogenicity and dissemination to close bacteria of those resistant genes. Strains lacking an inducible AmpC-type β-lactamase that overproduces their chromosomal β-lactamase has been often linked to a plasmid-encoded AmpC-type enzyme but others mechanisms can lead to similar resistance

phenotypes (Philippon et al., 2002). Isolate TO01E, from a tomato purchased at a street market, conforms to this assumption as presenting a constitutive AmpC phenotype with stable β-lactamase hyperproduction, but no plasmid-encoded AmpC-gene was detected.

Fortunately, ECC strains with hyperproduction of AmpC β-lactamase, similarly to other *Enterobacter* spp., generally remain susceptible to carbapenems. However, we report the detection of an *E. ludwigii* strain (AG32) that produces AmpC enzymes and is suspected to be a carbapenemase producer (MAST D72C set). Notably, this strain shared the ACT-54 variant with strain LE43E, and both showed an intermediate sensibility to imipenem. Some ECC strains have been observed to exhibit carbapenem resistance when associated with outer membrane permeability defects caused by a specific chromosome-encoded cephalosporinase (Jousset et al., 2019). Strain AG32 was isolated from irrigation water in a farm, which may facilitate its easy dissemination to fresh vegetables. Infections caused by such strains could result in therapeutic failure, as imipenem is considered the most effective antibiotic for treating *E. cloacae* infections (Davin-Regli et al., 2019).

Interestingly, two strains of *E. kobei* (AG07E and ZA03E) and another two ones of *E. ludwigii* (ES13E and LE07E), which were detected in vegetable and irrigation water samples, were phenotypically resistant to colistin, considered a last resort antibiotic. It must be highlighted that the strain AG07E obtained from irrigation water showed a MIC value as high as 128 mg/ml and harboured a mobile colistin resistance gene (*mcr-9.1*; Table 3). The detection of *mcr*-positive Enterobacteriaceae in vegetables remains limited, with only few reported cases. These include the identification of several *mcr-1*-positive *E. coli* strains on green leafy vegetables from Portugal (Manageiro et al., 2020) and Algeria (Chelaghma et al., 2022), two cases of imported vegetables in Switzerland (Zurfuh et al., 2016), and the presence of *mcr*-positive Enterobacteriaceae in specific vegetables in China (Liu et al., 2019; Luo et al., 2017). To the best of our knowledge, the presence of the *mcr-9* gene in ECC strains in vegetables has only been reported in one previous study conducted in the USA (Moon et al., 2022). In that study, the *mcr-9* gene was detected in strains of *E. hormaechei*; however, those strains did not exhibit resistance to colistin, indicating the gene may have been silenced. In contrast, our study identified four colistin-resistant ECC strains, among which the mobile *mcr-9* gene was detected in one *E. kobei* strain (AG07E). Importantly, we demonstrated the transferability of this gene through a plasmid-mediated mechanism. On the other hand, the four colistin-resistant strains harboured other chromosomal mechanisms such as the pmrHFIJKLM operon, *pmrE* and *pmrC* (*eptA*) genes, which have been associated with polymyxin resistance. These genes are naturally present in *Enterobacter* spp. However, it should be noted that the presence of these genetic determinants may vary among individual bacterial strains and not all bacteria of a species necessarily possess it. The development of resistant phenotypes has been linked to specific mutations in the genes that regulate the expression of these mechanisms, such as pmrA/pmrB and PhoP/PhoQ, resulting in constitutive overexpression (Olaitan et al., 2014). We found substitutions in pmrB for the two colistin-resistant *E. kobei* strains, as it was also reported by L. Xu, Wan, et al. (2022) on one human *E. kobei* isolate harbouring the *mcr-10* gene. The ZA03E strain also presented an additional amino acid substitution in pmrA. Of particular interest, this MDR *E. kobei* strain demonstrated *in vitro* adherence to human colon cells, indicating an enhanced potential for clinical risks. This finding is particularly significant considering the rising prevalence of *Enterobacterales* lacking sensitivity to colistin in humans over the past decades. The transmission of resistant isolates from animals to humans, acquisition via food and exposure to colistin in human treatments have been attributed to this increased prevalence (Binsker et al., 2022). In their review, Binsker et al. reported a regional prevalence of colistin resistance among human clinical *Enterobacterales* ranging from 2.4% to 3.4%, which is close to the global resistance rate of 3.6%. Among the resistant genera, *Enterobacter* spp. were found to be the most abundant. Thus, our results reveal high rates of colistin-resistant ECC isolates (16.7%), emphasising the need for monitoring this resistance in the enterobacterial group isolated from fresh vegetables and their production environment, particularly the water, under a One Health approach.

Bacterial pathogenesis encompasses a crucial initial stage of bacterial attachment to host cells. Among the investigated antimicrobial-resistant ECC strains, we observed that two *E. hormaechei* strains (LE47E and TO42E), along with the *E. kobei* ZA03E strain, demonstrating potential *in vitro* adherence to human colon epithelial cells. It is noteworthy that the *E. hormaechei* strains revealed a constitutive AmpC phenotype, exhibiting LE47E further hyperproduction of the b-lactamase and lacking sensibility to 4GC. ECC strains have been reported to produce extracellular protein fibres known as Curli, which play a crucial role in surface adhesion and invasion. In terms of pathogenicity, ECC strains have been shown to induce apoptosis in human Hep2 cells (Krzymińska et al., 2010).

E. kobei ZA03E demonstrated a remarkable ability to adhere to both HT-29 colon cell line and mouse fibroblasts. This particular ability to adhere to epithelial cells may be linked to the genomic island carried by this isolate. This region shares similarities with *serU* genomic island found in *E. coli* UPEC CFT073 (Spurbeck et al., 2011) and *Salmonella enterica* serovar Enteritidis ROD21 (Quiroz et al., 2011). The detection of this pathogenic island is of particular interest as it may contribute to adhesion to epithelial cells, possibly through *pilV* and Type IV pilus major pilin genes encoded within it (Strom & Lory, 1993).

A high degree of genetic diversity was observed among the analysed isolates using a 7-locus MLST scheme, which was further confirmed by PFGE analysis. Specifically, among the identified sequence types, ST13 and ST102 were the only ones present in multiple strains (two strains each), with ST13 (strains CI24E and PJ39E) being associated with the same pulsetype. This finding highlights the presence of clonal diversity within ECC, consistent with previous studies (Knecht et al., 2022). The MLST scheme, which utilises 7-housekeeping genes, is recognized for its ability to provide detailed classification of ECC isolates and demonstrate comparable or greater discriminative power compared to other molecular typing methods (Viau et al., 2017). Of particular interest is the assignment of the water strain AG07E to ST56, which has been predominantly reported in *E. kobei* strains isolated from human patients in China and known to harbour the *mcr-9* gene (Liao et al., 2022; T. Xu, Xue, et al., 2022). Notably, L. Xu, Wan, et al. (2022) reported that strains belonging to ST56 were part of the same clonal group as a carbapenem-resistant strain (*mcr-10*+) assigned to ST125. Our findings may support this observation as the MDR *E. kobei* strain ZA03E, obtained from carrot samples and exhibiting colistin resistance, also belonged to ST125, demonstrated pathogenic potential, and showed a close genetic relationship to the water strain AG07E.

5. Conclusions

Fresh vegetables and their cultivation environments appear to serve as significant reservoirs for ECC strains that produce AmpC β-lactamases. Our analysis underscores the genetic diversity observed among AmpC-resistant ECC strains obtained from agricultural settings, particularly revealing high rates of colistin-resistant isolates which is typically uncommon in this bacterial group. In particular, our study reports, for the first time, the detection of a colistin-resistant *E. kobei* strain (AG07E) in irrigation water from a vegetable farm in Spain. This strain carries the *mcr-9.1* gene and has the ability to transfer this resistance by conjugation. Interestingly, this strain belongs to the ST56 sequence type, along with other clinical *E. kobei* strains that also carry the *mcr-9* gene. Additionally, we identified the MDR-*E. kobei* strain ZA03, isolated from carrots obtained from a street market, which exhibited resistance to colistin, showed potential for human pathogenicity, and belonged to the ST125 sequence type, which has clonal relationships with strains in ST56. These findings emphasise the importance of monitoring and addressing the spread of antimicrobial-resistant ECC strains isolated from fresh vegetables and their production environment under a One

Health perspective, with particular attention to the role of irrigation water in their transmission.

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CRediT authorship contribution statement

Alberto Pintor-Cora: Software, Validation, Investigation, Data curation, Writing – review & editing, Visualization. **Ángel Alegría:** Software, Investigation, Visualization. **Jose Ramos-Vivas:** Data curation, Writing – review & editing, Supervision. **María-Luisa García-López:** Conceptualization, Validation. **Jesús A. Santos:** Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Funding acquisition. **Jose M. Rodríguez-Calleja:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used GPT-3.5 language model (OpenAI; https://platform.openai.com/models/gpt-3.5) in order to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115382.

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