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Biofilm formation ability and tolerance to food-associated stresses among ESBL-producing *Escherichia coli* strains from foods of animal origin and human patients

Paula Fernández-Gómez *, Elena Trigal, Ángel Alegría, Jesús A. Santos, Mercedes López, Miguel Prieto, Avelino Alvarez-Ordóñez

Department of Food Hygiene and Technology, Universidad de León, León, Spain

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ABSTRACT

The dissemination of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* continues to be an important health concern, with the food production chain as a suggested reservoir. A collection of 31 strains, including ESBL-producing *E. coli* strains isolated from foods of animal origin and from human patients and reference collection strains, was evaluated regarding their biofilm formation ability, tolerance to food-associated stresses (heat, acid, non-thermal atmospheric plasma (NTAP) and UV-C light) and RpoS status. The most relevant phenotypic differences among strains were observed for biofilm formation and heat resistance, being related to the source of isolation (clinical *vs* food *vs* reference) or to the sequence type (ST131 *vs* other STs), while only minor differences were related to the occurrence of the ESBL genes (bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV}). The biofilm formation ability on stainless steel was significantly higher for the field isolates, both clinical- and food-related, than for the reference strains. Food isolates were more thermotolerant (58 °C-1 and 2 min) than clinical isolates. Minor differences among strain categories were observed for their tolerance to the other inactivation technologies. Some polymorphisms in the *rpoS* gene sequences were detected, but loss-of-function mutations were not found, with the clustering of strains mainly based on their sequence type.

1. Introduction

The worldwide spread of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* constitutes a persistent challenge regarding therapies against multidrug resistant bacteria. ESBLs are β-lactamases able to hydrolase penicillins and cephalosporins, thus bacteria that harbour ESBL encoding genes become resistant to β-lactam agents (Chong, Shimoda, & Shimono, 2018; Doi, Iovleva, & Bonomo, 2017; Kawamura et al., 2017). Their epidemiology has evolved since their first detection in the 1980s, with increasing detection rates and the inclusion of ESBL-producing *Enterobacteriaceae* on the list of critical priority pathogens for the discovery of new antibiotics (World Health Organization, 2017). Even though a decreasing trend in prevalence has been observed since 2016, on the period 2019–2020 the mean prevalence values of presumptive ESBL producers on food-producing animals and derived meat for all EU member states were between 4.3%, on bovine meat, and 34.1%, in pigs, varying greatly by countries (EFSA, 2021).

One of the main sources for the spread of ESBL-producing bacteria into the community are food-producing animals, where the transmission of ESBL encoding genes via mobile genetic elements from animal associated *Enterobacteriaceae* to human pathogenic *E. coli* may occur (Bergšpica, Kaprou, Alexa, Prieto, & Alvarez-Ordóñez, 2020). In fact, ESBL-producing *E. coli* have been detected in food-producing animals in different European and non-European countries, although differences in experimental methodologies make it difficult to compare prevalence data among countries or sources of isolation (EFSA, 2011; Ramos et al., 2020). Also, their presence has been widely reported on foods of animal origin, which have been reported as a reservoir for the transmission of ESBL-producing *E. coli* within the human population (Alegría et al., 2020; Day et al., 2019; Kaesbohrer et al., 2019; Tekiner & Özpınar, 2016).

On food processing environments, the persistence of antibiotic-resistant bacteria, including ESBL-producing, can be enhanced within biofilms. The spatial structure of biofilms, with the bacteria embedded in a self-produced extracellular polymeric substances (EPS) matrix, reduces the fitness cost of antibiotic resistance as the resistant bacteria

* Corresponding author.
E-mail address: pafeg@unileon.es (P. Fernández-Gómez).

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compete with a reduced subpopulation in a close fixed proximity (France, Cornea, Kehlet-Delgado, & Forney, 2018). Additionally, biofilms are often regarded as hot spots for horizontal gene transfer since cell-to-cell contact facilitates the transmission of plasmids carrying antibiotic resistance genes, including ESBL genes (Maheshwari, Ahmad, & Althubiani, 2016; Stalder & Top, 2016).

The dissemination of antibiotic resistance in the food chain might be also accelerated due to co- or cross-resistance events involving various common food-associated stressors, such us low temperatures, acid pH, osmolytes and sanitizers. Specifically, for *E. coli*, cross protection to different antibiotics has been observed, for instance, after its exposition to acid pH (Mitosch, Rieckh, & Bollenbach, 2017) and UV treatments (Zhang, Xu, Wang, Zhuang, & Liu, 2017). One of the proposed molecular determinants responsible for that association in Gram-negative bacteria is the sigma factor σ^s (RpoS), a transcriptional regulator of the stationary phase and the general stress response encoded by the gene *rpoS* (Alvarez-Ordóñez, Broussolle, Colin, Nguyen-The, & Prieto, 2015; Fernández-Gómez, López, Prieto, González-Raurich, & Alvarez-Ordóñez, 2020), which might play an important role in bacterial cross-protection phenomena (Liao et al., 2020).

The aim of this work was to evaluate the biofilm formation ability as well as the tolerance to heat, acid pH, non-thermal atmospheric plasma (NTAP) and UV-C light of a collection of 31 *E. coli* strains, 27 ESBL-producing *E. coli* strains isolated from foods of animal origin and from human patients and 4 reference *E. coli* collection strains. Furthermore, possible associations between biofilm formation ability, resistance to food-associated stresses, RpoS status, carriage of different ESBL encoding genes, strain's MLST sequence type, and strain's isolation source were also investigated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study included 27 ESBL-producing *E.coli* isolates (12 from samples of food of animal origin and 15 from the Department of Clinical Microbiology of Complejo Asistencial Universitario de León (CAULE)), all of them previously characterized in a recent research article form our research team (Alegría et al., 2020). In addition, 4 *E. coli* reference strains from the Spanish Type Culture Collection (CECT) were included (Table 1). In the above mentioned study, ESBL-producing *E.coli* were isolated from samples of food of animal origin (chicken meat, ewe's milk and goat milk) in Chromagar ESBL (Chromagar, Paris, France) and confirmed as *E. coli* through Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI--TOF) identification. The presence of the ESBL genes bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} was assessed for all these isolates by PCR detection and the MLST sequence type determined by amplifying and sequencing seven conserved housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*), as recommended by the EnteroBase Database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and described in detail in Alegría et al. (2020).

Cultures were maintained in cryovials at −20 °C and recovered by streaking them in Brain Heart Infusion (BHI, Merck, Germany) agar plates, which were incubated at 37 °C for 24 h. Bacterial suspensions were then prepared by inoculating 10 mL of BHI broth with an isolated colony and incubating again at 37 °C for 24 h.

2.2. Assessment of biofilm formation on stainless steel and polystyrene

The biofilm formation ability of the 31 strains was evaluated on Ø 35 mm polystyrene plates (Thermo Scientific, USA) and on custom Ø 35 mm stainless steel AISI 316 plates, provided by the Department of Mechanical Engineering from University of La Rioja (Spain). The plates were inoculated with 4 mL of a 1:100 dilution on fresh BHI broth of the bacterial inocula (~10^6 CFU/mL) and incubated at 37 °C for 24 h.

Table 1
Genetic characteristics and isolation source of the ESBL-producing *E. coli* and reference *E. coli* strains included in the study.

Strain	Source	Sequence type	bla_{TEM}	$bla_{CTX\text{-}M}$	bla_{SHV}
#1	clinical	ST131		+	
#2	clinical	ST131		+	
#3	clinical	ST88	+	+	
#4	clinical	ST86		+	
#5	clinical	ST131	+	+	
#6	clinical	ST131	+	+	
#7	clinical	ST131		+	
#8	clinical	ST648	+		+
#9	clinical	ST131		+	
#10	clinical	ST131		+	
#11	clinical	ST131	+	+	
#12	clinical	ST131	+	+	
#13	clinical	ST117		+	
#14	clinical	ST131		+	
#15	clinical	ST131		+	
#16	food (chicken meat)	unknown	+		+
#17	food (ewe's milk)	ST57	+		
#18	food (ewe's milk)	ST447	+		
#19	food (chicken meat)	ST373	+		+
#20	food (ewe's milk)	ST57	+	+	
#21	food (ewe's farm)	unknown	+		
#22	food (goat's milk)	ST155	+	+	
#23	food (ewe's farm)	ST57	+		
#24	food (goat's milk)	ST59		+	
#25	food (ewe's milk)	ST57	+	+	
#26	food (chicken meat)	unknown			+
#27	food (ewe's milk)	ST13	+		
#28	reference (CECT99)	ST1079			
#29	reference (CECT515)	ST95			
#30	reference (CECT516)	ST3021			
#31	reference (CECT4267)	ST11			

+: presence of the ESBL gene assessed through PCR detection by Alegría et al. (2020).

Afterwards, the biofilms were washed three times by immersion in Ringer solution (Merck, Germany), stained for 15 min with 4 mL of 1% crystal violet (Panreac, Spain) and washed again three times with Ringer solution to remove the excess of stain. After a drying step, the plates were incubated with 5 mL of ethanol 96% for 15 min and the optical density (OD) at 595 nm of the ethanol solution was measured with a spectrophotometer (UV–3100PC, VWR, USA). Four replicates were made for each strain and plates with non-inoculated BHI media were included as negative controls.

Based on their ability to form biofilms either on SS or on polystyrene, the strains were classified into four categories following the approach previously described by Stepanović, Ćirković, Ranin, and Švabić-Vlahović (2004). Briefly, an OD cut-off value (ODc) was calculated as the mean OD plus three SD of the negative control. The strains were assigned, based on the measured OD, to one of the following categories: no-biofilm producer (OD < ODc), weak biofilm producer (ODc ≤ OD < 2 * ODc), moderate biofilm producer (2 * ODc ≤ OD < 4 * ODc) and strong biofilm producer (OD ≥ 4 * ODc).

2.3. Evaluation of bacterial inactivation under stress conditions

Heat and acid stress tolerance experiments were performed in PBS (G-Biosciences, USA) inoculated with the bacterial inoculum to achieve ~ 10^5 CFU/mL. The heat treatments were carried out in 10 mL of PBS previously stabilized at a temperature of 58 °C in a thermostatic water bath, inoculated with 0.1 mL of the appropriated bacterial inoculum dilution and samples (0.1 mL) were taken after 0, 1 and 2 min of heat exposition. The acid treatments were performed in 1 mL of PBS adjusted to pH 2.5 with HCl (Panreac, Spain) and samples (0.1 mL) were taken after 0, 15 and 30 min of incubation at room temperature. All the samples were spread-plated on BHI agar plates and incubated at 37 °C for 24 h.

Non-thermal atmospheric plasma (NTAP) and UV-C treatments were performed on BHI agar plates superficially inoculated with 0.1 mL of the appropriate dilution to reach a final bacterial density of ~10^3 CFU per plate. The inoculum was spread-out on the entire upper agar surface and samples were then allowed to dry for 15 min in a laminar flow cabinet (Telstar, model BV-100, P.A., USA) before UV-C and NTAP treatments were conducted. The resistance to NTAP was evaluated using a lab-scale plasma jet (CP121 Plasma Demonstrator OMVE BV, Netherlands) operated at atmospheric pressure and room temperature with air as working gas at a flow rate of 10 L/min, a voltage difference of 2 kV between electrodes and an output power of 1 W (Calvo, Álvarez-Ordóñez, Prieto, González-Raurich, & López, 2016). The UV-C treatments were applied using a UVPCX 2000 crosslinker (UVP, Upland, CA, USA) at a wavelength of 254 nm. Inoculated agar plates were treated with NTAP for 1 and 2 min and with UV-C light at two energy intensities, 2 and 6 mJ/cm^2. After the treatment, the plates were incubated for 24 h at 37 °C. Inoculated BHI agar plates not exposed to the treatments served as control.

In all stress-resistance experiments, two independent replicates were performed in different days. For all the inactivation treatments, the incubation of the plates was followed by the enumeration of survivors and the bactericidal effect was assessed by calculating log reductions using the following formula:

log reduction = - log (Nc/Nt), where:

Nc = CFU counts in untreated samples

Nt = CFU counts in treated samples

The treatment conditions and exposure times were selected based on the results obtained in previous studies by the group (Álvarez-Molina et al., 2020; Álvarez-Ordóñez et al., 2013).

2.4. Sequencing of the rpoS gene

A 1.3-kpb fragment containing the *rpoS* gene was amplified and sequenced with the primers RPOS-1 (AAGGCCAGCCTCGCTTGAG) and RPOS-3 (TGATTACCTGAGTGCCTACG), previously used by Álvarez-Ordóñez et al. (2013). PCR reactions (50 μL) were performed in a thermocycler ProFlex PCR System (Applied Biosystems) using the DreamTaq DNA polymerase kit (Thermo Scientific), 0.4 μM of each primer and 1 μL of template DNA. The PCR program included 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The PCR products were analysed by electrophoresis in a 1% p/v agarose gel (Sigma, St. Louis, Missouri, EE. UU) and purified using the NucleoSpin Gel and PCR Clean-up kits (Macherey-Nagel). Sequencing was performed using an automatic capillary ABI 3500 Genetic Analyzer (Applied Biosystems) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), following the instructions of the manufacturer. Only in 23 of the 31 strains it was possible to sequence the complete length of the target gene. The forward and reverse sequences were merged using the online EMBOSS suite of bioinformatics tools (http://emboss.sourceforge.net/). Multiple local alignments of the DNA sequences, the translation to amino acids and the alignment of the amino acid sequences was carried out with Geneious (v 2021.2.2). The online multiple alignment program MAFFT version 7 (https://mafft.cbrc.jp/alignment/software) was used to build rooted neighbour-joining phylogenetic trees that were displayed including phenotypic information with R Studio (v 4.1.1). The likely effect of single amino acid polymorphisms was predicted with the bioinformatic tools PROVEAN (Choi, Sims, Murphy, Miller, & Chan, 2012) and SIFT (Ng & Henikoff, 2003), with a PROVEAN score lower than −2.5 and a SIFT score lower than 0.05 indicating a deleterious change.

2.5. Statistical analysis

Data regarding biofilm formation (4 replicas) and inactivation (2 replicas) of the *E. coli* strains was analysed after grouping them based on the isolation source (food, clinical or reference), presence or absence of bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} genes, and sequence type identification (ST131 *vs* other sequence types) in order to find associations between these variables and the strains' phenotypes. Mean differences among the categories in the different variables were analysed by using the Student's *t*-test for independent samples and considered statistically significant at $p < 0.05$. The statistical analyses were performed with R Studio (v 4.1.1).

3. Results

The relationship between the source of isolation, sequence type and carriage of ESBL encoding genes with particular phenotypic characteristics (i.e., biofilm formation ability and resistance to food-related stresses) of a collection of 31 *E. coli* strains (4 reference strains, 15 ESBL-producing strains of clinical origin and 12 ESBL-producing strains of food origin) was evaluated. Results obtained for each individual strain are reported in Fig. S1.

3.1. Biofilm formation

As illustrated in Fig. 1A, the biofilm formation ability on stainless steel was significantly higher for the field isolates, both clinical- and food-related, than for the reference strains included in the study. On this surface, 3.2% of the strains were classified as no-biofilm producers (n = 0 clinical; 0 food; 1 reference), 38.7% as weak biofilm producers (n = 5 clinical; 5 food; 2 reference), 54.8% as moderate biofilm producers (n = 10 clinical; 6 food; 1 reference) and 3.2% as strong biofilm producers (n = 0 clinical; 1 food; 0 reference). However, no significant differences among isolation source categories were found for biofilm formation ability on polystyrene, where 6.5% of the strains were classified as no-biofilm producers (n = 0 clinical; 2 food; 0 reference), 9.7% as weak biofilm producers (n = 2 clinical; 0 food; 1 reference), 48.4% as moderate biofilm producers (n = 10 clinical; 4 food; 1 reference) and 35.5% as strong biofilm producers (n = 3 clinical; 6 food; 2 reference).

Furthermore, biofilm formation on both SS and polystyrene was statistically lower for the isolates that harbour the bla_{SHV} gene, present in only 4 of the studied strains, than for those that do not carry it (Fig. 1E), and significantly higher, just on SS, for the isolates that harbour the bla_{TEM} gene than for the rest of *E. coli* strains (Fig. 1C).

3.2. Heat resistance

Clinical isolates were significantly more sensitive to the heat treatments at 58 °C for 1 and 2 min than food-related and reference strains, with log reductions from 0.13 ± 0.02 to 0.26 ± 0.04 and 0.16 ± 0.04 to 0.40 ± 0.09 for reference strains; from 0.00 ± 0.02 to 0.42 ± 0.12 and from 0.14 ± 0.21 to 0.48 ± 0.13 for food isolates; and from 0.12 ± 0.01 to 0.540 ± 0.002 and 0.26 ± 0.02 to 0.78 ± 0.11 for clinical isolates, respectively (Fig. 2A).

When the strains were grouped based on their sequence type, ST131 isolates were also more heat sensitive than strains from other sequence types (Fig. 2B). ST131 strains showed mean log reductions of 0.38 ± 0.12 and 0.56 ± 0.15 while the strains assigned to other STs showed log reductions of 0.22 ± 0.13 and 0.31 ± 0.17 for the 1-min and 2-min treatment at 58 °C, respectively. It is important to note that most strains belonging to ST131 were of clinical origin, while none of the ST131 strains originated from food products (Table 1). Finally, the most noticeable association with the presence of ESBL encoding genes was a lower resistance to heat by strains harbouring the $bla_{CTX\text{-}M}$ gene (Fig. 2D). In this regard, it is important to note that the distribution of bla_{TEM} and $bla_{CTX\text{-}M}$ genes on the strains was not homogeneous among the different isolation sources. Thus, 83% of the food isolates carried the bla_{TEM} gene compared to 40% of the clinical isolates, and the $bla_{CTX\text{-}M}$ gene was detected in 93% of the clinical isolates but only in 33% of the food isolates. This fact could explain why both the clinical isolates and

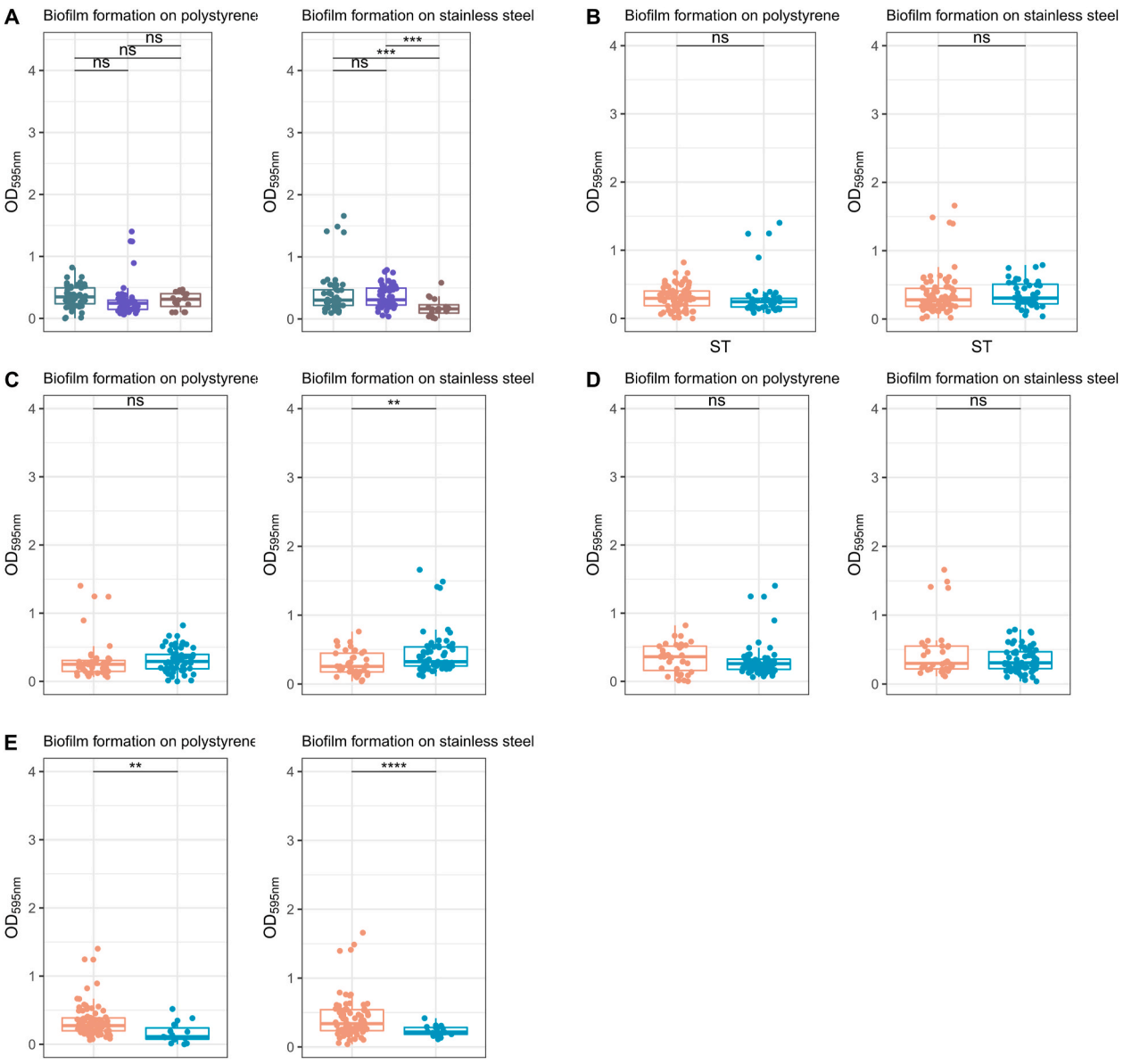

Fig. 1. Phenotypical characterization results regarding biofilm formation obtained for the ESBL-producing *E. coli* strains grouped based on their (A) source of isolation (food (●) *vs* clinical (●) *vs* reference (●)), (B) ST (ST131 (●) *vs* non-ST131 (●)) and presence (●) or absence (●) of (C) bla_{TEM}, (D) $bla_{CTX\text{-}M}$ and (E) bla_{SHV} genes. Asterisks indicate statistically significant differences between the two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

the strains which harboured the $bla_{CTX\text{-}M}$ gene were significantly more sensitive to thermal treatments than their counterparts.

3.3. Acid resistance

Regarding tolerance to acid pH, clinical isolates were slightly more sensitive to the exposition for 30 min to an extreme pH value than the food related ones (Fig. 3A), and no further statistical differences among strain categories were observed. This suggests that there are no strong associations between the strains ST or the carriage of ESBL-encoding genes and *E. coli* susceptibility to low pH. Overall, the observed log reductions where between -0.07 ± 0.05 and 0.14 ± 0.02 for the shorter 15-min treatment and between 0.03 ± 0.01 and 0.95 ± 0.13 for the longer 30-min treatment.

3.4. NTAP resistance

Minor intraspecific NTAP resistance variability was detected, with log reductions that ranged from 0.43 ± 0.08 to 1.43 ± 0.34 for the 1 min NTAP exposure treatment and from 1.11 ± 0.26 to 2.99 ± 0.49 for the 2-min NTAP exposure treatment. Only strains harbouring the gene bla_{TEM} showed more resistance to exposition to plasma for 1 min than their counterparts, with 0.69 ± 0.19 *vs* 0.90 ± 0.33 log reductions, respectively (Fig. 4C).

3.5. UV-C light resistance

The bacterial inactivation achieved with UV-C light treatments was higher than with the heat, acid pH and NTAP treatments under the selected conditions. Indeed, the log reductions achieved ranged from 0.19 ± 0.03 to 2.57 ± 0.34 for the 2 mJ/cm^2 treatment and from 0.76 ± 0.23 to >3 for the 6 mJ/cm^2 treatment. The results from UV-C light treatments undertaken at 6 mJ/cm^2 (Fig. S1) were excluded from the statistical analyses since in most cases survivor counts were below the limit of quantification of the analytical method (>3.0 log reductions). The inactivation achieved by the UV-C light treatments at 2 mJ/cm^2

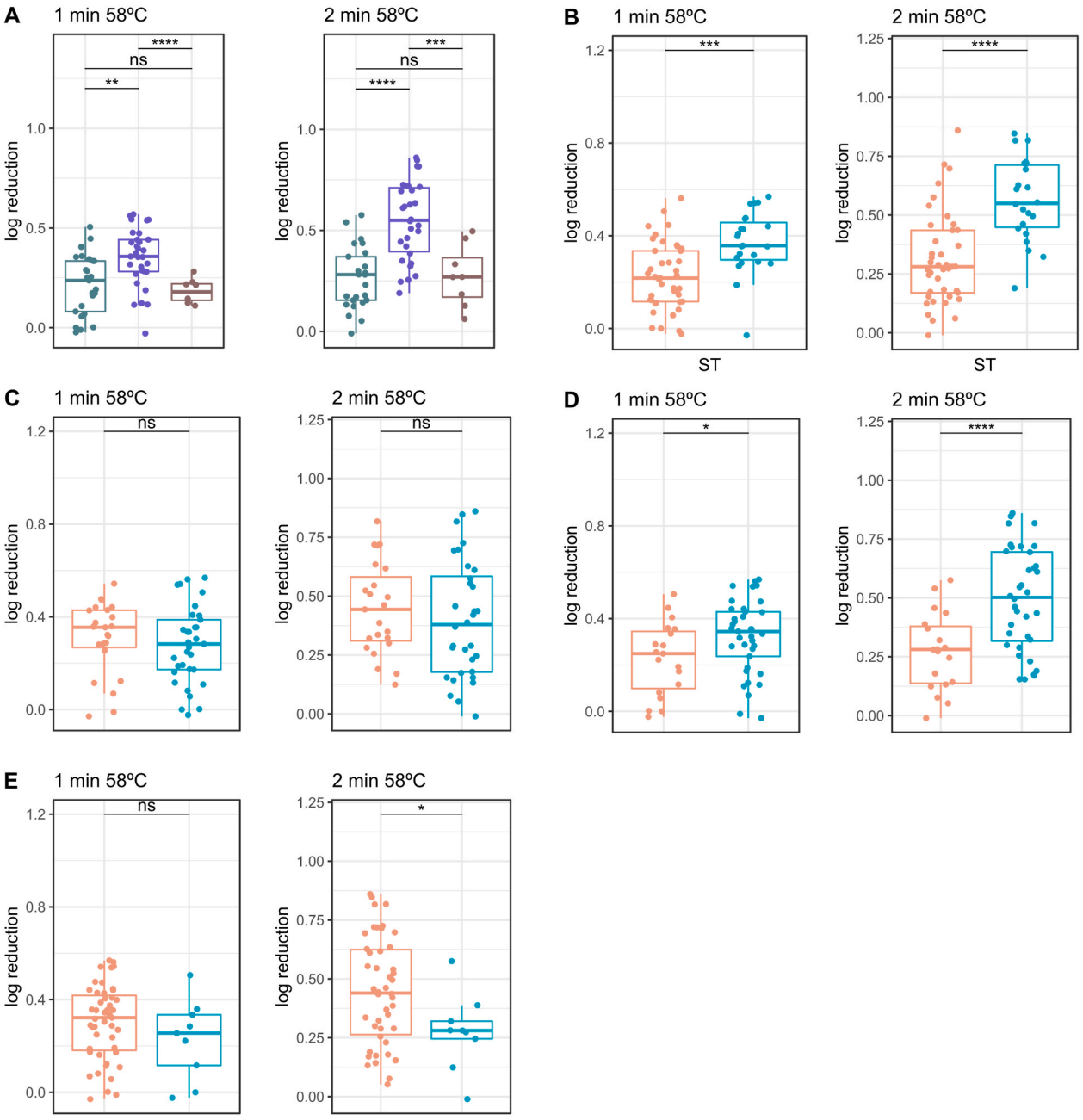

Fig. 2. Phenotypical characterization results regarding inactivation after exposure to heat obtained for the ESBL-producing *E. coli* strains grouped based on their (A) source of isolation (food (●) *vs* clinical (●) *vs* reference (●)), (B) ST (ST131 (●) *vs* non-ST131 (●)) and presence (●) or absence (●) of (C) bla_{TEM}, (D) $bla_{CTX\text{-}M}$ and (E) bla_{SHV} genes. Asterisks indicate statistically significant differences between the two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

showed only minor differences between the analysed groups (Fig. 5). Indeed, statistically significant differences were only observed for the carriage of bla_{TEM}, with strains harbouring this gene showing lower resistance to 2 mJ/cm^2 UV-C light treatments (Fig. 5C).

3.6. Global analysis

A global analysis of *E. coli* biofilm formation ability and stress tolerance was performed by assigning to the strains values ranging from 0, for the most stress sensitive or least biofilm former strain, to 10, for the most stress resistant or most biofilm former strain, and adding the values obtained for all studied variables to obtain an index of the strain's resilience or ability to persist. No associations ($p > 0.05$) with the sequence type and carriage of ESBL encoding genes were found (Fig. 6).

However, isolates from food were more resilient, with a mean global index of 55.08 ± 4.35, followed by reference strains (51.43 ± 4.98) and the clinical strains (44.87 ± 10.03), although statistical differences were only detected between food and clinical isolates. None of the evaluated strains presented consistently the highest stress resistance and biofilm formation ability or the highest stress sensitivity and lower biofilm formation ability. Nevertheless, some strains could be identified as multisensitive, such as the strains #14, #3 and #8, all of clinical origin, that showed the lowest global index values, and as multiresistant, like the strains #15, #20 and #26, all isolated from food, that showed the highest ones (**Fig. S2**).

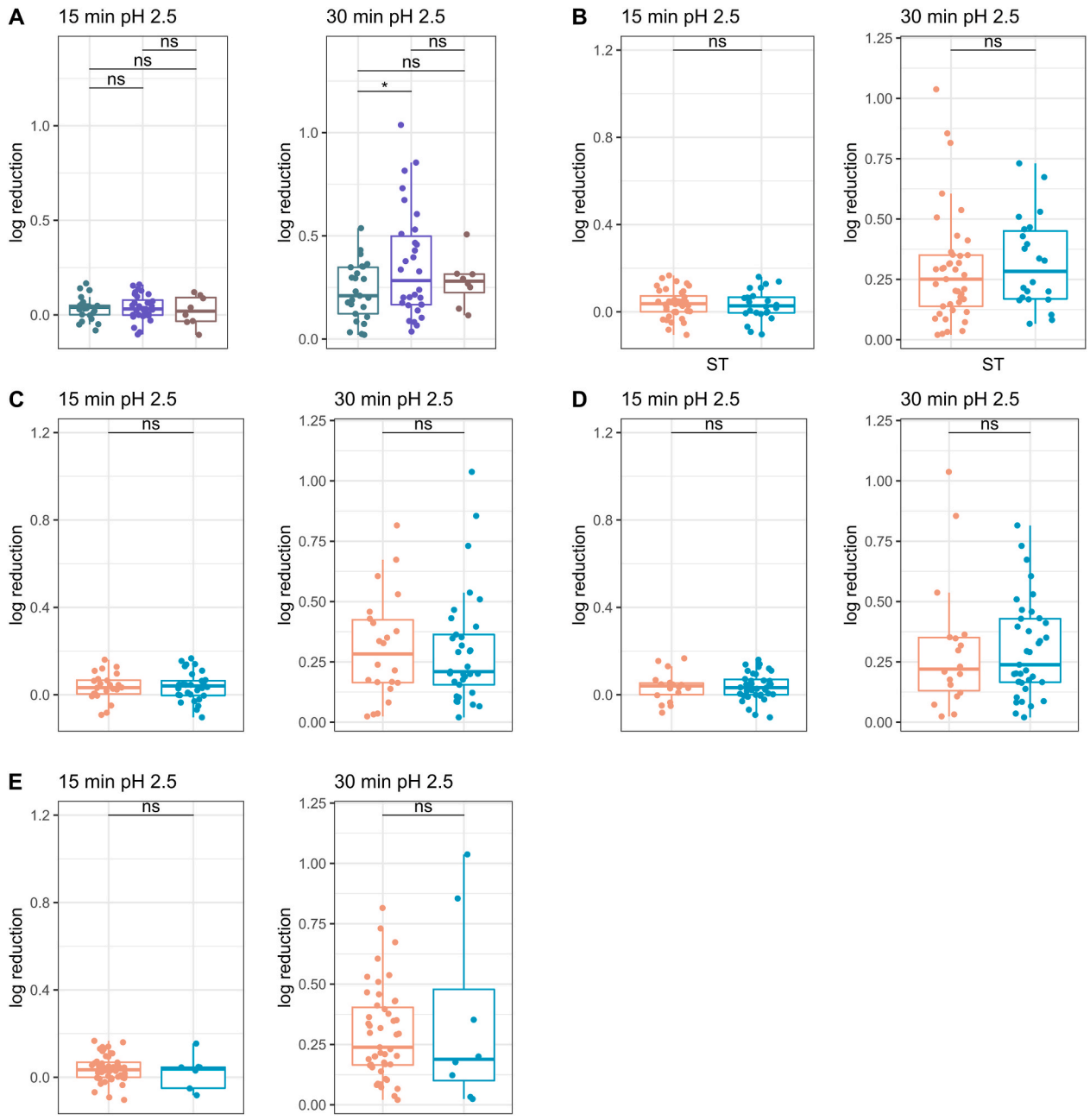

Fig. 3. Phenotypical characterization results regarding inactivation after exposure to acid pH obtained for the ESBL-producing *E. coli* strains grouped based on their (A) source of isolation (food (●) *vs* clinical (●) *vs* reference (●)), (B) ST (ST131 (●) *vs* non-ST131 (●)) and presence (●) or absence (●) of (C) bla_{TEM}, (D) $bla_{CTX\text{-}M}$ and (E) bla_{SHV} genes. Asterisks indicate statistically significant differences between the two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

3.7. RpoS polymorphisms

Variations in the *rpoS* gene sequence are described in Table 2. A total of 11 positions with single-nucleotide polymorphisms (SNPs) were identified in the 23 strains for which the *rpoS* gene could be amplified and fully sequenced. 9 out of the 11 identified SNPs were synonymous SNPs and did not translate into changes in the amino acid sequence. Nevertheless, the other 2 SNPs were non-synonymous and resulted in amino acid substitutions of different relevance. The analysis of this amino acid changes revealed that the substitutions observed at positions 188 (lysine to asparagine) and 311 (arginine to cysteine) can be classified as non-conservative changes (PROVEAN score of −4.1 and −5.7 and SIFT score of 0.01 and 0, respectively), but none of the strains with these single amino acid substitutions showed notable different phenotypic characteristics. No loss-of-function mutations (i.e., leading to a truncated protein) were detected. In fact, the phylogenetic trees constructed with the *rpoS* nucleotide sequence showed no clear relation between the polymorphic variations in the *rpoS* gene and the biofilm formation ability or resistance to food-related stresses (acid, heat, NTAP or UV-C light) of the strains (Data not shown). However, it was possible to observe clustering based on the sequence type (ST131 *vs* other STs) and the source of isolation (Food *vs* Clinical *vs* Reference), as shown in Fig. 7.

4. Discussion

The close association between the worldwide spread of ESBL-producing resistant bacteria and the intensive use of β-lactam antibiotics in both veterinary and human medicine has led to numerous studies focused on investigating the possible transmission routes of

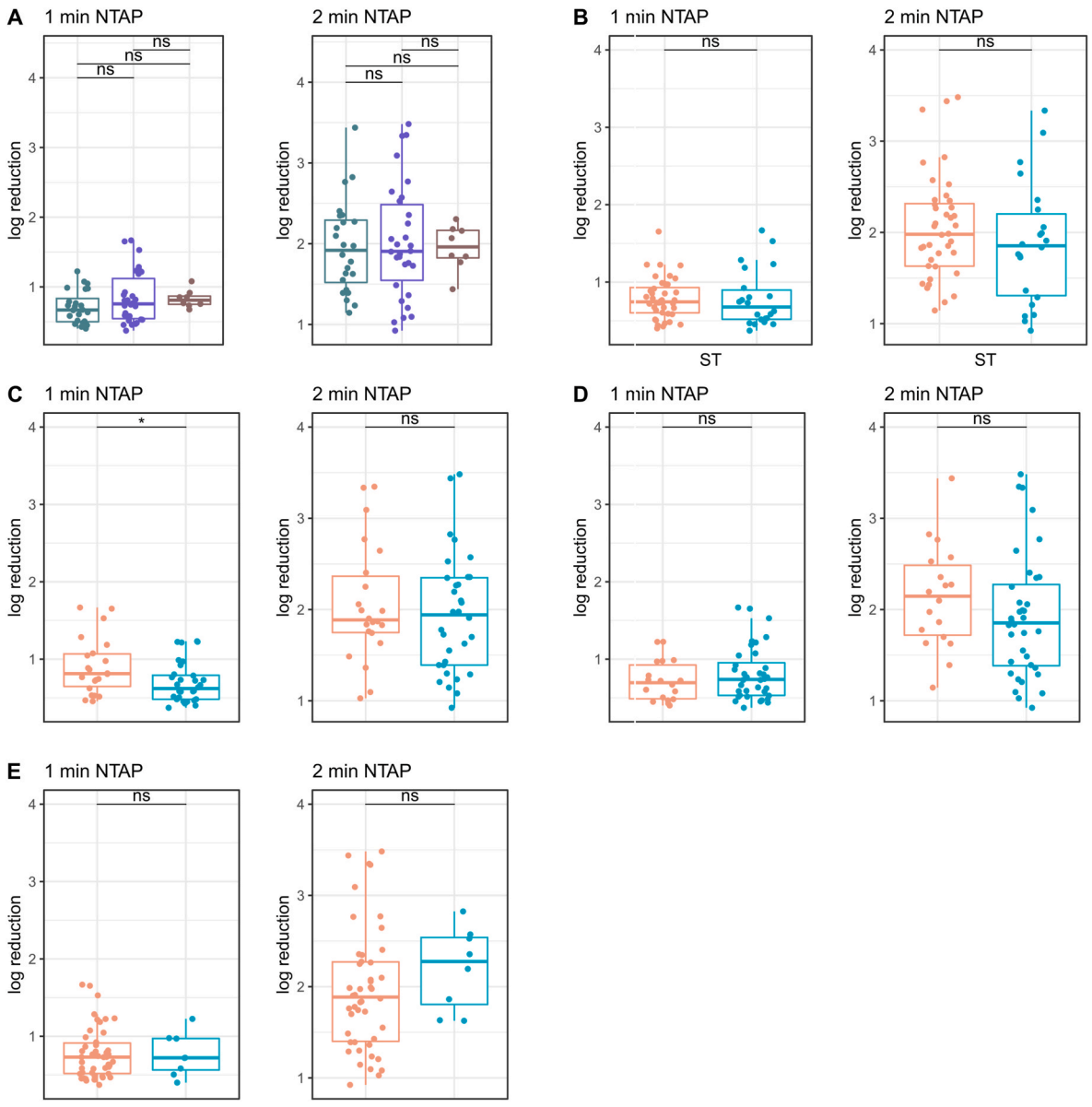

Fig. 4. Phenotypical characterization results regarding inactivation after exposure to NTAP obtained for the ESBL-producing *E. coli* strains grouped based on their (A) source of isolation (food (●) *vs* clinical (●) *vs* reference (●)), (B) ST (ST131 (●) *vs* non-ST131 (●)) and presence (●) or absence (●) of (C) bla_{TEM}, (D) $bla_{CTX\text{-}M}$ and (E) bla_{SHV} genes Asterisks indicate statistically significant differences between the two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

ESBL-producing *E.coli* from livestock to humans (Day et al., 2019; EFSA, 2011; Ewers et al., 2021; Horigan, Kosmider, Horton, Randall, & Simons, 2016; Kurittu et al., 2021; Ludden et al., 2019; Pérez-Rodríguez & Mercanoglu Taban, 2019). The current study is a follow-up investigation which continues on a previously published molecular characterization of a collection of ESBL-producing *E. coli* isolates from food (milk and chicken meat) and clinical origin (Alegría et al., 2020). Here we deepen in the phenotypic characterization of this collection of isolates by exploring their potential to tolerate food-associated stress conditions and to form biofilms on stainless steel and polystyrene.

The results showed limited phenotypic heterogeneity among the tested strains, with most differences being related to the isolation origin and/or sequence type rather than to the carriage of ESBL encoding genes. The similarities in the results obtained for clinical isolates and for ST131 isolates as well as for food isolates and other STs, especially in the case of heat tolerance, could be, at least in part, explained by the predominance of ST131 among the strains isolated from human patients, while it was absent among the strains isolated from foods. The high prevalence of this sequence type among hospital-derived ESBL-producing *E. coli* isolates is in agreement with the widely described rapid worldwide dissemination of ST131 *E. coli*, which is believed to represent from 22 to 66% of the human clinical ESBL-producing *E. coli* isolates in Europe (Chong et al., 2018; Nicolas-Chanoine, Bertrand, & Madec, 2014).

Regarding biofilm formation ability, some differences were observed between groups of ESBL-producing *E. coli* isolates based on the source of isolation, sequence type or presence of the genes bla_{TEM}, $bla_{CTX\text{-}M}$ or bla_{SHV}. Although on a collection of *E. coli* and *Klebsiella pneumoniae* ESBL-producing strains it has been previously reported that high levels of biofilm formation are less frequently observed among ST131 clones, it is also discussed that the general diversity of ST131 strains might affect the results obtained (Surgers, Boyd, Girard, Arlet, & Decré, 2019). On the other hand, a tranconjugation study showed that ST131 isolates with plasmids harbouring $bla_{CTX\text{-}M\text{-}14}$ *or* $bla_{CTX\text{-}M\text{-}15}$ demonstrated a diminished level of biofilm formation even though their growth rate and fitness was similar to those observed for their host strains (Shin & Ko,

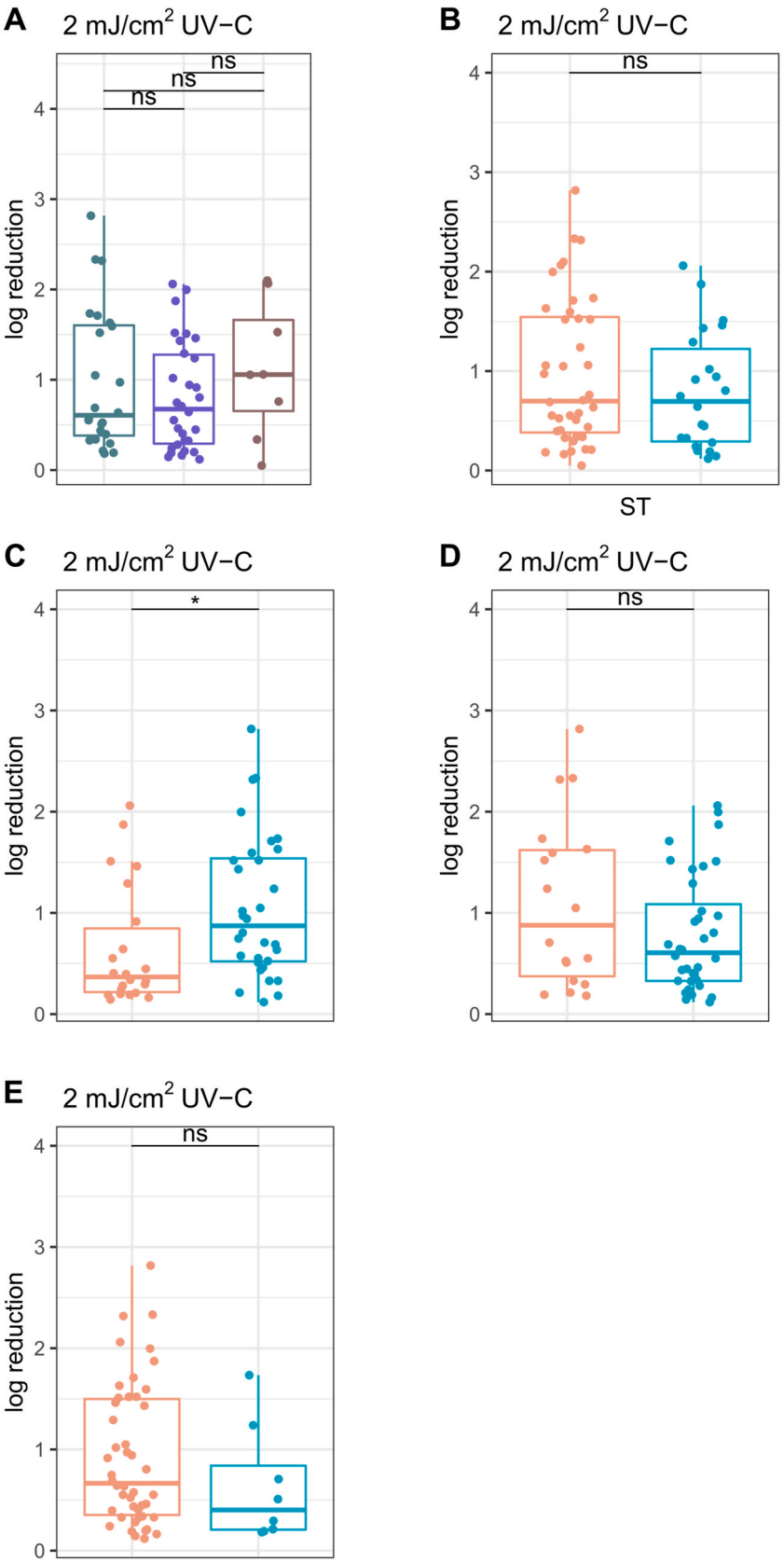

Fig. 5. Phenotypical characterization results regarding inactivation after exposure to UV-C light obtained for the ESBL-producing *E. coli* strains grouped based on their (A) source of isolation (food (●) *vs* clinical (●) *vs* reference (●)), (B) ST (ST131 (●) *vs* non-ST131 (●)) and presence (●) or absence (●) of (C) bla_{TEM}, (D) $bla_{CTX\text{-}M}$ and (E) bla_{SHV} genes. Asterisks indicate statistically significant differences between the two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

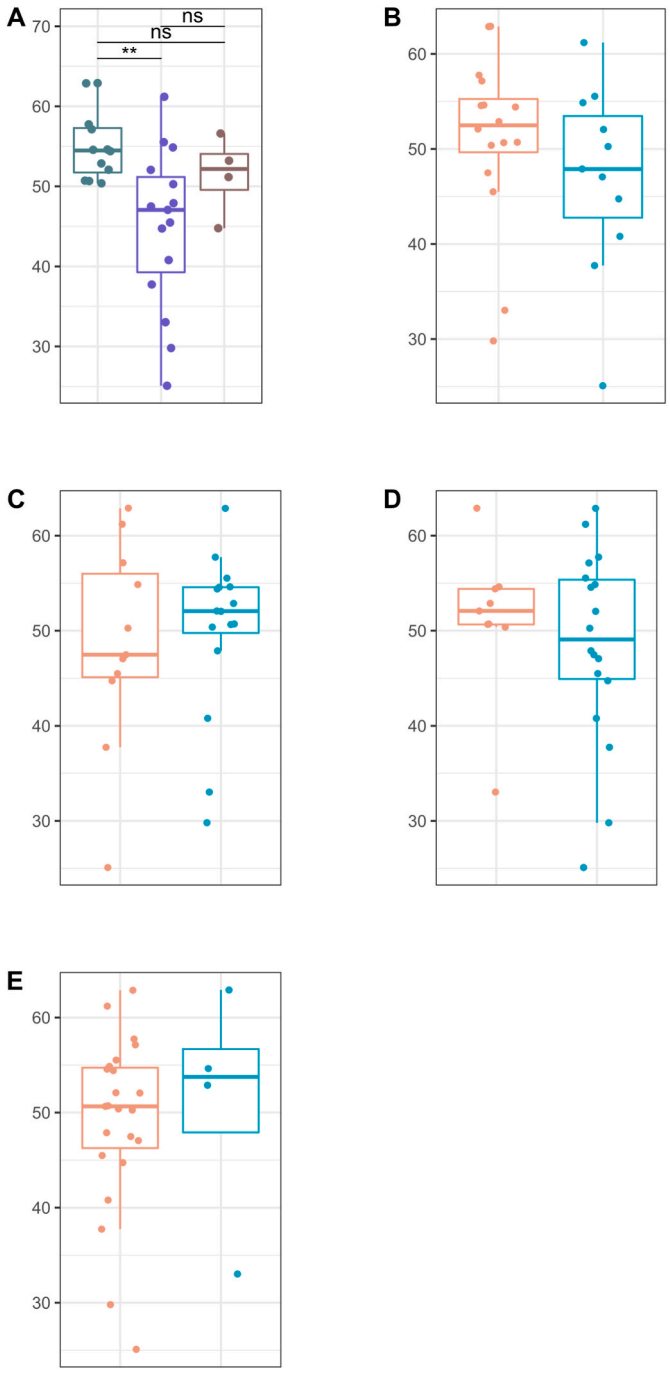

Fig. 6. Global analysis of acid, heat, PANT and UV-C light resistance and biofilm-forming ability. For each condition, strains were assigned a numeric value, ranging from 0 (for the most stress sensitive or least biofilm former strain) to 10 (for the most stress resistant or most biofilm former strain), taking into account the log reductions (for the stress resistance experiments) or the OD measurements (for the biofilm-forming experiments). ESBL-producing *E. coli* strains were grouped based on their (A) source of isolation (food (●) *vs* clinical (●) *vs* reference ([●])), (B) ST (ST131 (●) *vs* non-ST131 (●)) and presence (●) or absence (●) of (C) bla_{TEM}, (D) $bla_{CTX\text{-}M}$ and (E) bla_{SHV} genes. Asterisks indicate statistically significant differences between the two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

2015). However, in the current study the main difference in biofilm formation ability was found between field (food and clinical) and reference strains, on stainless steel, which might be linked to the frequent exposure of field isolates to disinfection protocols at hospitals or food processing plants that may favour the selection of strains more capable to form biofilms and thus evade sanitation.

To detect possible associations between ESBL production and tolerance to food-related stresses the bacterial inactivation caused by the exposure to NTAP, acid, heat and UV-C light treatments was evaluated for the strain collection. The cross protection effect between resistance to some antibiotics and tolerance of bacteria to multiple food-associated stresses has been previously described (Giacometti, Shirzad-Aski, & Ferreira, 2021; Liao et al., 2020). Even though it has been less studied specifically for ESBL-producing *E. coli*, these associations have been previously suggested for other microorganisms. For *Staphylococcus aureus*, a similar tolerance to plasma-activated water (PAW) has been observed between a methicillin-resistant *S. aureus* (MRSA) and a methicillin-susceptible *S. aureus* (MSSA) (Wang, Han, Liao, & Ding, 2021) and a similar or higher tolerance to acid, heat and osmotic stress for antibiotic-resistant and multi-antibiotic resistant (MAR) *S. aureus* (Ma et al., 2019). Similar results have been found for *L. monocytogenes*, with MAR strains being less susceptible those food-related stresses (Komora, Bruschi, Magalhães, Ferreira, & Teixeira, 2017), while for *E. coli* MAR strains showed a lower tolerance to heat and acid stress (Duffy, Walsh, Blair, & McDowell, 2006), In the current study statistical differences were mainly observed only for heat resistance, with clinical isolates being more easily inactivated than food and reference strains. These differences may indicate that food processing can select more robust strains, with higher ability to tolerate moderate thermal treatments. The potential of heat-resistant *E. coli* to act as a reservoir of plasmids encoding ESBL on the dairy industry has been studied in the past (Marti et al., 2016). These authors reported that the high fraction of heat-resistant isolates, together with the observed ability of strains carrying ESBL plasmids to act as donor strains for conjugation, increase the possibility of ESBL-producing heat resistant strains becoming more abundant in these environments. Also, a cluster of strains showing increased thermoresistance has been identified in a collection of Danish ESBL-producing *E. coli* isolates from hospital patients, being highlighted that this characteristic might facilitate their survival on medical devices exposed to mild high temperatures (Boll, Frimodt-Møller, Olesen, Krogfelt, & Struve, 2016).

On the other hand, only minor differences among strain groups were observed for the UV-C light treatments, results that agree with the previously described lack of differences on the inactivation of ESBL-producing and no-ESBL-producing *E. coli* with UVA-LED technology (Ulfa et al., 2020). Similarly, the tolerance of the studied *E. coli* strains to NTAP was strain-dependent and it was not associated either with the carriage of ESBL-genes, the source of isolation or the ST. Even though, to our knowledge, there are no previous publications comparing the tolerance to NTAPof ESBL-producing and no-ESBL-producing *E. coli*, the inactivation levels achieved in the current study were higher than those obtained for *E. coli* in previous studies by our research group in food matrices (Calvo, Prieto, Alvarez-Ordóñez, & López, 2020), but similar to those found on surfaces by Cahill et al. (2014).

The fitness cost of ESBL-plasmid-carriage might explain some of the phenotypic differences observed in the current study. The carriage of plasmids is generally considered a fitness burden, but multiple publications in recent years have shown that some specific ESBL-carrying plasmids in *E. coli* ST131 and ST648 lineages enhance virulence without affecting fitness (Dimitriu et al., 2019; Ranjan et al., 2018; Schaufler et al., 2016). In the current study, isolates harbouring the bla_{SVH} and $bla_{CTX\text{-}M}$ genes showed lower biofilm formation abilities and heat resistance, respectively, suggesting that they might be less competitive in some stressful environments prevailing in the food chain. However, as it has been previously discussed, clinical ST131 isolates harbour more frequently the $bla_{CTX\text{-}M}$ gene, which makes it difficult to conclude if the observed effect is the result of the fitness cost of carrying these genes or if it is more associated with the higher robustness of the strains from other ST, that are prevalent in the food industry and therefore adapted to the stresses characteristic of food processing environments.

Table 2
Polymorphisms in the rpoS gene and their effect on the translated amino acid sequence for the 23 sequenced strains.

Strain	Base (amino acid) at position:										
	163-165 (55)	355-357 (119)	460-462 (154)	562-564 (188)	565-567 (189)	571-573 (191)	697-699 (233)	730-732 (244)	817-819 (273)	931-933 (311)	940-942 (314)
#1	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#2	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#3	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTG (V)	CGC (R)	CGT (R)
#5	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTG (V)	CGC (R)	CGC (R)
#6	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#7	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#9	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#10	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#11	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#12	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#14	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#15	CTG (L)	CTG (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#16	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAC (H)	GCG (A)	AAC (N)	GTG (V)	CGC (R)	CGT (R)
#17	TTG (L)	CTT (L)	ATT (N)	**AAT (N)**	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTA (V)	CGC (R)	CGC (R)
#18	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTG (V)	CGC (R)	CGC (R)
#19	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTG (V)	CGC (R)	CGT (R)
#20	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTA (V)	CGC (R)	CGC (R)
#21	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCA (A)	AAC (N)	GTA (V)	**TGC (C)**	CGC (R)
#22	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTG (V)	CGC (R)	CGC (R)
#23	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTA (V)	**TGC (C)**	CGC (R)
#25	TTG (L)	CTT (L)	ATT (N)	AAG (K)	CTG (L)	CAT (H)	GCG (A)	AAC (N)	GTA (V)	CGC (R)	CGC (R)
#30	TTG (L)	CTT (L)	ATC (N)	AAG (K)	CTG (L)	CAC (H)	GCA (A)	AAT (N)	GTG (V)	CGC (R)	CGC (R)
#26	TTG (L)	CTT (L)	ATT (N)	**AAT (N)**	TTG (L)	CAT (H)	GCG (A)	AAC (N)	GTG (V)	**TGC (C)**	CGC (R)

A = Alanine, C = Cysteine, H = Histidine, K = Lysine, L = Leucine, N = Asparagine, R = Arginine.
Non-synonymous single nucleotide polymorphisms (nsSNPs) are highlighted in bold.

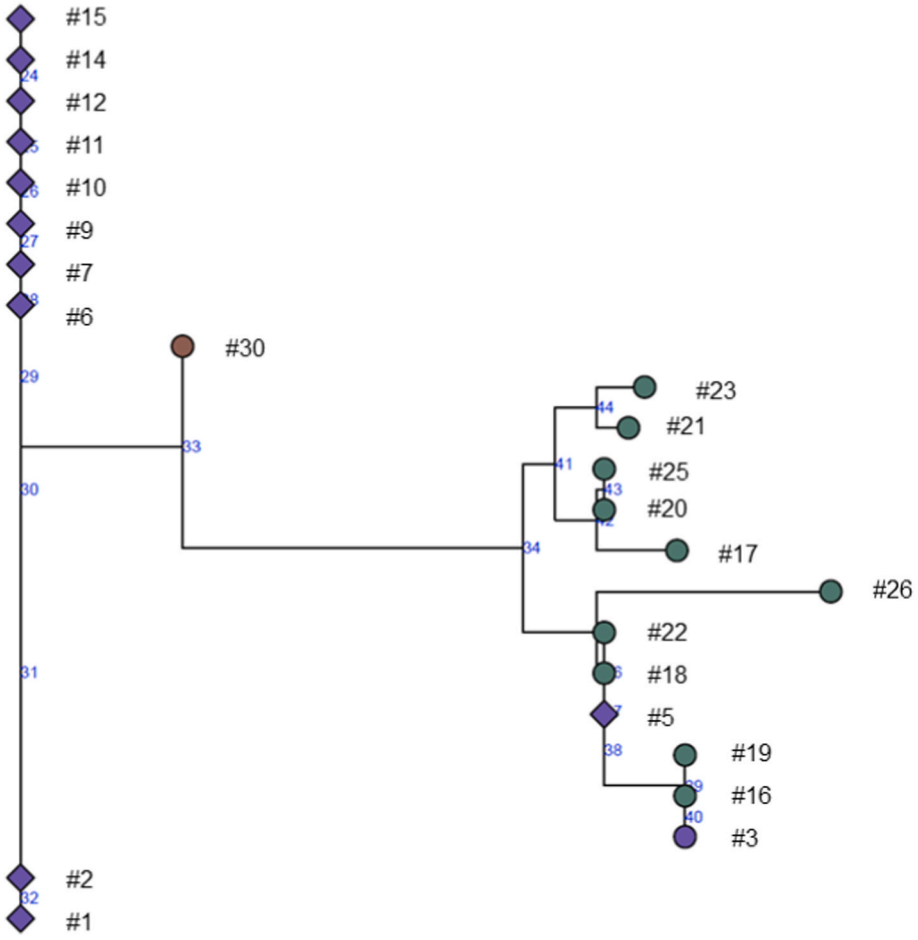

Fig. 7. Rooted neighbour-joining phylogenetic tree of the *E. coli rpoS* gene sequences, including information on the isolation source (food (■) *vs* clinical (■) *vs* reference (■)) and sequence type (ST131 (◇) *vs* non-ST131 (○)).

A screening of the RpoS status of the *E. coli* strains in the collection was performed to evaluate the possible role of this sigma factor transcriptional regulator in the associations between antibiotic resistance and tolerance to food-associated stress conditions, as previously suggested by Liao et al. (2020). The *rpoS* gene was sequenced for 23 out of the 31 strains and only minor variations in the nucleotides sequence were observed, which would translate into amino acid substitutions in only 2 positions and for only 4 strains. The absence of loss-of-function mutations could partially explain the lack of variability observed in the tolerance of the strain collection to different food-related stressors. Previous studies showing *E. coli* phenotypic variability associated with the RpoS status included strains with loss-of-function mutations in this gene (Álvarez-Ordóñez et al., 2013; Robey et al., 2001; Waterman & Small, 1996). This mutation has been found in natural *E. coli* populations related with phenotypes of lower resistance to high hydrostatic pressure, with a frequency of 2 out of 11 evaluated strains (Robey et al., 2001), and of high sensitivity to alkaline and heat treatments as well as lower ability to establish mature biofilms, with a frequency of 2 out of 12 evaluated strains (Álvarez-Ordóñez et al., 2013). Also, Waterman and Small (1996) identify 13 acid-sensitive strains in a collection of 58 *E. coli* isolates and observed that the introduction a plasmid carrying the *rpoS* gene conferred acid resistance to the majority of them. In the present study, the phylogenetic trees built showed *rpoS* sequence similarities according mainly to the sequence type of the strain, with isolates of clinical origin (mainly from ST131) clustering separately from isolates of food origin (from a range of other STs). This association was again related to the widely described predominance of ST131 among hospital-derived ESBL-producing *E. coli* (Chong et al., 2018; Nicolas-Chanoine et al., 2014).

In conclusion, no strong associations between the presence of bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} genes and biofilm formation ability or resistance to food-associated stresses (heat, acid, NTAP and UV-C light) were found in the evaluated collection of ESBL-producing and reference *E. coli* strains. However, biofilm formation ability was higher on field isolates than on reference strains, and clinical isolates showed lower tolerance to heat than food isolates and reference strains. Similar associations were found when strains were categorised based on their sequence type (ST131 *vs* other sequence types), with ST131 strains, specifically linked to clinical samples and related to the worldwide abundance of ST131 on hospital settings, being also more heat sensitive than strains from other STs. Further studies are needed to better understand the role of the food chain on the spread of ESBL-producing *E. coli* and the molecular base of their resistance to food-related stresses.

CRediT authorship contribution statement

Paula Fernández-Gómez: Investigation, Formal analysis, Writing – original draft. **Elena Trigal:** Investigation. **Ángel Alegría:** Investigation, Writing – review & editing. **Jesús A. Santos:** Resources, Writing – review & editing, Funding acquisition. **Mercedes López:** Visualization, Writing – review & editing, Supervision. **Miguel Prieto:** Visualization, Writing – review & editing, Supervision. **Avelino Alvarez-Ordóñez:** Formal analysis, Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Available as Supplementary data

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Cristina Albalá Díez and Sandra Rodríguez de la Torre were involved in the performance of some of the laboratory experiments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113961.

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