International Journal of Food Microbiology 413 (2024) 110605

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Comparative analysis of multidrug-resistant *Klebsiella pneumoniae* strains of food and human origin reveals overlapping populations

Sergio Silva-Bea [a], Manuel Romero [a], Ana Parga [a], Javier Fernández [b,c,d], Azucena Mora [e,f,1], Ana Otero [a,*,1]

[a] *Department of Microbiology and Parasitology, Faculty of Biology, Universidade de Santiago de Compostela, Santiago de Compostela, Spain*
[b] *Servicio de Microbiología, Hospital Universitario Central de Asturias (HUCA), 33011 Oviedo, Spain*
[c] *Grupo de Microbiología Traslacional, Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), 33011 Oviedo, Spain*
[d] *Research & Innovation, Artificial Intelligence and Statistical Department, Pragmatech AI Solutions, Spain*
[e] *Laboratorio de Referencia de Escherichia coli (LREC), Department of Microbiology and Parasitology, Universidade de Santiago de Compostela, Lugo, Spain*
[f] *Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS), 15706 Santiago, Spain*

ARTICLE INFO

Keywords:
ESBL
Zoonotic
Biofilm
Serum resistance
Hypermucoviscosity

ABSTRACT

Given the increasing incidence of multidrug-resistant (MDR) *Klebsiella pneumoniae* infections, it is of great interest to investigate the risk of transmission associated with the prevalence of this pathogen. Some studies have described fresh raw poultry meat as a reservoir of MDR *K. pneumoniae*, including clinically relevant sequence types (ST) and extended-spectrum β-lactamase (ESBL) strains, indicating possible consumer exposure. This study compared 47 MDR strains of *K. pneumoniae* from poultry meat and human clinical isolates to assess similarities, including analysis of antimicrobial resistance profiles and virulence factors involved in infection. In addition, several biofilm culture methods were evaluated for reproducible assessment of biofilm formation in *K. pneumoniae* strains. Globally, no association between strain origin and STs, hypermucoviscosity, biofilm formation or serum resistance could be found between isolates of food and clinical origin, nor an associated AMR pattern, suggesting overlapping populations. We found that LB supplemented with glucose in microaerobiosis was the best discrimination condition for biofilm formation in the active attachment biofilm cultivation model. The biofilm formation capacity was strongly dependent on culture conditions, with a strain-specific response, but only a minor increase in biofilm levels was recorded in clinical *K. pneumoniae* populations. Our results suggest that a similar risk of zoonosis transmission from potentially virulent foodborne strains previously observed in *E. coli* is also present in this high-priority pathogen. This study further confirms that foodborne isolates of *K. pneumoniae* pose a risk to consumers and therefore this pathogen should be included in the surveillance of foodborne pathogens with high risk of MDR infections and therapeutic failure.

1. Introduction

Antimicrobial resistance (AMR) has been prioritised by the World Health Organization (WHO) as one of the top 10 global public health threats facing humanity (WHO, 2023). Moreover, in July 2022, the EU Commission and the Member States identified AMR as one of the top three priorities for human health (Council of the European Union, 2023). It has been estimated that bacterial AMR was directly responsible for 1.27 million global deaths and contributed to 4.95 million deaths worldwide in 2019 (Murray et al., 2022), and without intervention, multidrug-resistant (MDR) bacteria could cause 10 million deaths annually by 2050 (O'Neill, 2016). ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) are listed

---

Abbreviations: HMV, Hypermucoviscosity; ESBL, Extended-Spectrum β-Lactamase; AST, Antimicrobial susceptibility testing; MLST, Multi-Locus Sequence Typing; PFGE, Pulse Field Gel Electrophoresis; UPGMA, Unweighted Pair Group Method with Arithmetic mean; AA, active attachment; CV, Crystal Violet; PCA, Principal Component Analysis; DSBs, Double-Strand Breaks; AMR, Anti-Microbial Resistance; MDR, Multi-Drug Resistant; KP, *Klebsiella pneumoniae*; AE, aerobiosis; µAE, microaerobiosis.

[*] Corresponding author at: Universidade de Santiago de Compostela – Faculty of Biology, CIBUS building, 15706 Santiago de Compostela, A Coruña, Spain.
E-mail address: anamaria.otero@usc.es (A. Otero).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijfoodmicro.2024.110605
Received 25 September 2023; Received in revised form 8 January 2024; Accepted 22 January 2024
Available online 23 January 2024

within the WHO priority pathogens list for which new antibiotics are urgently needed (WHO, 2017). These pathogens are increasingly reported in food, where their ability to form biofilms poses a challenge to the food industry (Patil et al., 2021).

Among ESKAPE bacteria, *K. pneumoniae* (KP), a common intestinal bacterium implicated in nosocomial and community extraintestinal infections, caused up to 600,000 deaths in 2019 worldwide (Murray et al., 2022). Furthermore, in the context of the pandemic caused by SARS-CoV-2, KP caused at least 55 % of nosocomial respiratory co-infections in COVID-19 patients, being the most frequent complication of hospitalised COVID-19 patients and increasing the risk of death by 2.5-fold (García-Meniño et al., 2021). The latest report on AMR surveillance in Europe, jointly published by the European Centre for Disease Prevention and Control (ECDC) and the WHO Regional Office for Europe, based on data from 2021, shows that third-generation cephalosporin resistance in KP has become quite widespread in the WHO European Region (ECDC & WHO, 2023). Given the occurrence of KP MDR infections, the study of the risk of transmission involved in the prevalence of this pathogen is of great interest. A high similarity of commensal KP strains to community-acquired and nosocomial isolates have been reported, suggesting that potentially pathogenic strains may live in the gut microbiota silently as reservoirs (Gómez et al., 2021; Sequeira et al., 2020). On the other hand, *K. pneumoniae* has been newly suggested as a foodborne pathogen, together with extraintestinal pathogenic *E. coli* (Riley, 2020).

Increasing evidence points to zoonotic transmission as a major route of persistence and transmission of MDR strains. The presence of potentially pathogenic MDR-*E. coli* in poultry samples is well documented (Abreu-Salinas et al., 2020; García et al., 2023; McLellan et al., 2018) but assessing only MDR-*E. coli* in meat may underestimate contamination with other MDR strains. KP MDR strains carrying $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{TEM}, bla_{SHV}, bla_{CMY} and plasmid-mediated quinolone resistance genes have been described in the environment, animals and food (Aslam et al., 2022; Wareth and Neubauer, 2021; Wu et al., 2022; Zhang et al., 2018). In fact, the presence of antimicrobial-resistant KP strains in food samples, including extended spectrum β-lactamase (ESBL) strains, has been reported in many countries by the Agency for Food Safety and Nutrition (AESAN) (Franco Abuín et al., 2023), highlighting the exposure of consumers to these potentially pathogenic strains. Indeed, KP, although not included in the WHO zoonoses inventory, has been reported as a zoonotic disease in several publications (Hu et al., 2021; Santaniello et al., 2020; Soto et al., 2012) and has been included in the European Centre for Disease Prevention and Control inventory as “other potential zoonotic agents” (EFSA and ECDC, 2023). Phylogenetic analyses suggest close relationships between human- and livestock-derived KP, as STs associated with hypervirulent lineages have been isolated from raw retail turkey meat (Díaz-Jiménez et al., 2020). In addition, isolates from meat were significantly more likely to be MDR than clinical isolates, probably reflecting the selective pressures of antibiotic use in animal food production. Furthermore, isolates from human and animal sources showed similar virulence in a murine sepsis model (Holt et al., 2015).

Key KP virulence factors implicated in infections, such as the hypermucoviscous phenotype (HMV), iron-binding siderophore production, as well as lipopolysaccharide production and capsular hyperproduction (González-Ferrer et al., 2021) are of great interest to assess potential zoonotic transmission risk. Capsular hyperproduction is one of the most important virulence factors of KP, highly correlated with increased mortality, as it leads to increased resistance to human serum, phagocytosis and antibody opsonisation (Guerra et al., 2022). Biofilm formation is also a key virulence factor contributing to the persistence of infections since it impairs antimicrobial action and provides an optimal environment for horizontal transmission of antibiotic resistance genes (González-Ferrer et al., 2021; Guerra et al., 2022). Additionally, the ability to form biofilm is associated with the persistence of MDR-KP species on food industry-related surfaces (González-Rivas et al., 2018). Given this, one would expect biofilm-forming bacteria to display resistance to multiple antimicrobials, a hypothesis supported by a study in which 73 % of MDR-KP clinical strains were reported as biofilm-hyperforming bacteria (Ramos-Vivas et al., 2019). In contrast, Cusumano et al. (2019) found that MDR-KP were 91 % less likely to form strong biofilm. A possible explanation for these discrepancies is the experimental methodology used to quantify biofilm formation, as this phenotype is strongly determined by the culture conditions, leading to different conclusions depending on the methodology used (García et al., 2023; Parga et al., 2023). Therefore, studying and comparing food and clinical-origin strains regarding this phenotype is of high interest to assess the potential zoonotic risk.

This study aims to compare KP strains of meat and human-clinical origin to assess similarities and potential foodborne transmission risk based on the analysis of genotypic and phenotypic traits, including AMR profiles and hypervirulence-related phenotypes such as HMV, biofilm formation and serum resistance, a phenotype previously linked to capsule production (Walker and Miller, 2020). To establish a reproducible methodology to assess biofilm formation in KP, we evaluated various biofilm culture methods, as well as different culture conditions known to affect biofilm formation and capsule production, such as oxygen availability and glucose supplementation. Studying the differences between the two populations allowed us to assess a better approximation of the zoonotic risk of transmission of MDR—*K. pneumoniae* strains, indirectly reflecting the emergence of high-risk-convergent strains.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of 47 ESBL- and/or carbapenemase-producing KP strains constituted the studied collection (Supplementary Table S1), which included 28 strains of meat origin and 19 of human clinical origin. The 28 meat strains were previously obtained from a random sampling of 100 fresh poultry samples (50 chicken and 50 turkey) every two weeks in 7 different supermarket chains in the city of Lugo (Spain), between September 2016 and September 2017. The 7 supermarket chains are representative of the national territory and the meat sampled was all from national producers. Of the 100 meat samples, 50 were chicken breasts and another 50 were turkeys. The meat products were transported in an isothermal container and processed within 2 h of collection. Briefly, 25 g of meat were aseptically cut from different parts of each sample, homogenised in 225 mL buffered peptone water and incubated for 6 h/37 °C. Then 1 mL of the pre-enriched sample was inoculated into 9 mL of MacConkey broth (18-24 h/37 °C). From the positive tubes, 10 μL each were plated on CHROMID® ESBL and CHROMID®CARBA SMARTmedia (BioMérieux) (18-24 h/37 °C). These media were used for the screening of ESBL- and carbapenemase-producing Enterobacteriaceae, respectively (Díaz-Jiménez et al., 2020). The 19 non-duplicated human KP isolates were recovered from different patients with different nosocomial infections (Supplementary Table S1) at the Hospital Universitario Central de Asturias (HUCA), Oviedo, in Northern Spain, whose health area covers a population of approximately 300,000 persons, between 2015 and 2019. The criterion used for selection was the therapeutic challenge posed by the infections, due to the AMR nature of the strains, which had been recovered by conventional culture in CHROMID™ CPS® Elite (BioMérieux, Marcy L'Étoile, France). In addition, the antibiotic-susceptible strain KP ATCC 13883^{T} was included as a reference strain. All strains were confirmed as KP by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany). The strain collection was stored in cryotubes (Scharlau, REF: 064-TA8275) at −80 °C and KP strains were routinely grown at 37 °C in lysogeny broth (LB) or LB agar (1.5 %).

2.2. Antimicrobial resistance susceptibility and genetic characterization of β-lactamase and colistin resistance genes

Antimicrobial susceptibility testing (AST) was performed by disc diffusion assay and included ampicillin, amoxicillin-clavulanic acid, cefuroxime, ceftazidime, cefotaxime, cefoxitin, aztreonam, imipenem, gentamicin, tobramycin, amikacin, fosfomycin, colistin, doxycycline, chloramphenicol, nitrofurantoin, co-trimoxazole, ciprofloxacin, nalidixic acid and tigecycline (Supplementary Table S1). All results were interpreted according to the Clinical and Laboratory Standards Institute guidelines, except for tigecycline, for which the clinical standard breakpoint established in 2022 by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2022) was applied. Based on the AST results, isolates were classified as MDR if they showed resistance to at least one agent from three or more antimicrobial categories (Magiorakos et al., 2012). The collection was also investigated by PCR for the screening of specific β-lactamase (*bla*) genes (IMP, VIM, KPC, OXA, TEM, CIT, SHV, CTX-M-1 and CTX-M-9 groups), as well as for plasmid-mediated colistin resistance (*mcr*) genes (1 to 5), using the primers and conditions described elsewhere (Díaz-Jiménez et al., 2020; Poirel et al., 2011).

2.3. Multilocus sequence typing (MLST)

The sequence type (ST) of the *K. pneumoniae* strains was determined following the Institute Pasteur MLST scheme (Diancourt et al., 2005).

2.4. Pulsed-field gel electrophoresis (PFGE)

For molecular subtyping of the collection, *Xba*I-PFGE profiles (macrorestriction profiles) were obtained following the standardised protocol PulseNet (Ribot et al., 2006), and imported into BioNumerics (Applied Maths, STMartens-Latern Belgium) to obtain a dendrogram using the UPGMA (unweighted pair group method with arithmetic mean) algorithm based on Dice's similarity coefficient and applying a 1 % tolerance in the position of the bands.

2.5. Hypermucoviscous phenotype

Hypermucoviscous phenotyping (HMV) was performed on the 48 strains following the string test with modifications (Shon et al., 2013). Bacteria were considered HMV+ when a slimy string ≥ 5 mm was formed when a colony was picked on an agar plate with a loop. The string tests were performed on colonies obtained on LB agar, glucose-supplemented LB (0.4 %), Columbia agar (5 % sheep blood) and EMB-Levine agar plates, all incubated in aerobiosis (AE) and microaerobiosis (μAE) (Campy GEN 2.5 L. REF: CN0025A) at 37 °C/24 h.

2.6. Biofilm cultivation and quantification

Biofilm-forming capacity was assessed in 24 representative strains (12 each of human and food origin), which were selected based on STs (clinically important and prevalent), PFGE macrorestriction profiles and origin (where possible, both origins were included). Experiments were also performed with the antibiotic sensitive ATCC 13883^T type strain. Biofilm culture was performed using the active attachment (AA) method as described in (Muras et al., 2020), which ensures that the biofilm is due to active cell attachment only and not deposition, and allows easy changing of culture media without disturbing the biofilm. Briefly, biofilms were grown in 12-well cell culture plates (VWR, REF: 734–2778) with wells filled with 3 mL LB or LB medium supplemented with 0.4 % glucose (LB+G), using a custom-made aluminium lid with glass coverslips (18x18mm) attached and partially immersed as biofilm substrate. Media were inoculated with cells at optical density at 600 nm (Abs_{600}) of 0.05 and incubated 24 h/37 °C under AE or μAE. The culture media were changed after 12 h to promote the growth of adherent cells. A total of 4 conditions were tested: LB AE, LB μAE, LB+G AE and LB+G μAE. All cultures were performed in triplicate. Biofilm biomass was quantified by staining with 0.04 % Crystal Violet (CV), washed once with distilled water, dried and CV was dissolved with 33 % acetic acid and quantified spectrophotometrically at 590 nm. Bacterial growth in the culture supernatant (Abs_{600}) was quantified spectrophotometrically to normalise the biofilm quantification data to total bacterial growth.

Additionally, the biofilm formation was studied aerobically for 7 strains using the real-time biofilm monitoring equipment xCELLigence® (RTCA DP Analyzer, Model 3 × 16), in which biofilm is measured as cell index due to changes in impedance values (Junka et al., 2012). Inoculum were prepared as for the AA method and media used included both LB and LB+G. Biofilm growth was monitored in 16-well *E*-plates (Aligent, REF: 5469830001) 24 h/37 °C. 3 sample replicates were carried out for each strain and condition tested. Real time data was processed with RTCA Software, version 2.0.0.1301.

A recently described new type of biofilm formation, termed R-biofilm (Liu et al., 2020), was also investigated in the same 24 strains selected for the AA method. This method assesses the formation of ring-shaped deposits in the culture tubes of KP after incubation in LB supplemented with the antibiotic bleomycin. Cultures (Abs_{600} 0.05) were incubated vertically in LB in aerobiosis 24 h/37 °C with the antibiotic bleomycin (40 μg/mL). A strain was considered positive for R-biofilm when a ring-shaped structure appeared in the tube, and this structure was able to withstand a flip of the tube without rupturing. In addition, the effect of DNase I (Roche) at 50 μg/mL was studied on the R-biofilm positive strains. These experiments were repeated twice. In parallel, growth of KP strains in the presence to bleomycin or DNase was assessed in triplicate.

2.7. Serum resistance

To assess the sensitivity of the KP isolates to human serum (Lv et al., 2022), microaerophilic overnights of each strain in LB or LB+G adjusted at Abs_{600} 1 were diluted 1/3 with human serum (Merk) and incubated aerobically for 2 h/37 °C in a 96-well U-bottom plate. As negative control, the inoculum was mixed with PBS pH 7.4. Subsequently, 1/10 serial dilutions were made in PBS and colony forming units per millilitre (CFU/mL) were quantified on LB agar. Three replicates were performed for each strain and condition.

2.8. Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.3.0 (GraphPad, San Diego, CA, USA). Initially, the data were tested for normal distribution using the Shapiro-Wilk test. If a normal distribution was found, ANOVA or t-Student tests were applied; otherwise, Kruskal-Wallis or Mann-Whitney analyses were performed, depending on whether >2 groups or only 2 groups were compared. Significance values represented in the graphs of this paper as asterisks are as follows: $* = p < 0.05$; $** = p < 0.005$; $*** = p < 0.0005$; and $**** = p < 0.00005$. Principal component analyses (PCA) for the AMR profile data were performed using RStudio version 2021.09.0.

3. Results

3.1. Genetic and resistance analyses reveal no significant differences between populations

Sequence types, macrorestriction profiles and AMR characterization were assessed to establish differences between human and food populations. Twenty-eight KP strains were recovered from 27 different meat samples. Significantly, only 2 isolates were from chicken (different samples) compared to 26 isolates from turkey (25 different samples). The 28 meat strains recovered on CHROMID® ESBL medium were phenotypically confirmed as ESBL-producers and showed MDR

(Supplementary Table S1). All meat strains expressed penicillin resistance; most (>80 %) showed resistance to cefotaxime, ciprofloxacin, cotrimoxazole, nalidixic acid; >60 % exhibited resistance to doxycycline, tigecycline; while none of them showed resistance against to cefoxitin, imipenem, amikacin or nitrofurantoin. (Fig. 1A). The ESBL typing revealed the simultaneous presence of $bla_{\text{CTX-M-15}}$ + bla_{SHV} in 13 of the 28 meat strains. All 28 meat strains were PCR negative for the presence of plasmid-mediated mobile colistin resistance genes (*mcr-1* to *mcr-5*), including the 5 strains phenotypically resistant to colistin. MLST revealed 11 different STs, although 6 accounted for 79 % of the 28 strains: ST307 (7), ST147 (4), ST4028 (4), ST15 (3), ST45 (2), ST111 (2). It is of note that ST307, ST147 and ST15 had been previously related as high-risk clones (Peirano et al., 2020).

In addition to ampicillin, most (>80 %) of the 19 KP from human clinical samples were resistant to amoxicillin-clavulanic acid, ciprofloxacin, nalidixic acid; and >60 % showed resistance to cefuroxime, ceftazidime, cefotaxime, aztreonam, gentamicin, doxycycline, nitrofurantoin, co-trimoxazole, tigecycline (Fig. 1A). All but one strain showed MDR (KLEB-41, ST4387). The ESBL typing determined the presence of $bla_{\text{OXA-48}}$ in 17 strains, 7 of which were also carriers of $bla_{\text{CTX-M-15}}$ alone, and 5 strains were simultaneously carriers of $bla_{\text{OXA-48}}$, $bla_{\text{CTX-M-15}}$ and bla_{SHV} (3 SHV-28 and 2 SHV-12) (Supplementary Table S1, Fig. S1). PCR screening of *mcr* genes (1 to 5) detected *mcr-1* in a phenotypically colistin-resistant KP CTX-M-15/SHV-28 clinical strain. The 19 human isolates belonged to 9 different STs: ST147 (6), ST326 (4), ST15 (2), ST405 (2), ST14 (1), ST16 (1), ST45 (1), ST307 (1), ST4287 (1).

All KP strains used in this study (including the ATCC 13883$^{\text{T}}$ control strain), showed 41 macrorestriction profiles that clustered in the *Xba*I-PFGE dendrogram according to their ST, except for some strains of ST45 and ST147 that were separated by origin. Ten clusters of similarity ≥85 % were observed, however, none of them included strains from both origins (Fig. 3: clusters highlighted in red). The XbaI-PFGE dendrogram showed >80 % identity for ST147 (4 strains) and ST307 (7 strains), and >76 % for ST15 (3 strains). PCA showed that both populations overlapped in terms of antibiotic profile resistance, although a greater dispersion was found among human isolates (Fig. 1B). PCA also highlighted chloramphenicol, aztreonam, ceftazidime and cefotaxime as those antibiotics most likely to discriminate human and food origin within our collection (Fig. 1C). PCA of the AMR profile was also performed for strains grouped according to their hypermucoviscous and R-biofilm phenotypes, as well as by ST, with no differences found between the groups analysed.

3.2. Hypermucoviscous phenotype is not correlated with the origin

The HMV phenotype, a virulence factor related to clinical infections (Shon et al., 2013), was studied in 8 different culture conditions (LB, LB+G, Columbia agar and EMB-Levine, all in AE and μAE) in both populations. Of all 48 strains studied, only 7 strains, 5 from meat origin and 2 from clinical samples, were HMV+ in at least one of the conditions tested (Table 1). The HMV phenotype was highly dependent on culture conditions, with only 2 meat strains (KLEB-21 and KLEB-22) maintaining the HMV+ phenotype in all conditions tested, with high expression of the phenotype on EMB-Levine and LB+G agars. The best discriminating conditions were LB+G μAE, LB+G AE and LB μAE, where the positive phenotype was expressed by the highest number of strains (Table 1). No correlation was observed between HMV phenotype and ST, PFGE profile, AMR or strain origin (Fig. 3).

3.3. Active attachment biofilm-cultivation method and population comparison

Considering the discrepancies found in the bibliography, we evaluated the biofilm formation of KP strains in two different biofilm-quantification models to subsequently compare both meat and human populations with the highest reproducibility method. First, biofilm formation was measured under aerobic conditions using the xCELLigence® real-time biofilm formation monitoring system with 7 KP strains in LB without or with glucose supplementation (LB+G 0.4 %). Glucose supplementation has been shown to significantly increase biofilm biomass and antibiotic tolerance in *E. coli* (Herzberg et al., 2006; Padilla et al., 2010). However, some authors have reported that glucose supplementation promotes the expression of genes related to capsule production through cAMP-catabolite repression in KP, and this could interfere with surface adhesins causing reduced biofilm formation (Chen et al., 2020). This prompted us to study the effect of glucose supplementation in biofilm cultures. A large variability between experimental replicates was observed using the xCELLigence® system (Supplementary Fig. S2). Faster cell attachment, indicated by a rapid increase in cell index, was observed in LB medium compared to LB+G cultures (Fig. 2C and Supplementary Fig. S2). In addition, all strains tested showed a drop in cell index values after 5 h of incubation, which was more acute in LB, probably indicating cell detachment, even reaching negative values after 10-15 h of incubation (Supplementary Fig. S2).

The low reproducibility of the results obtained with the xCELLigence® system prompted us to evaluate other biofilm culture systems in KP to more accurately discriminate the biofilm-forming capacity of strains. We therefore tested the AA biofilm culture method, previously reported to produce reproducible results in human pathogenic species (Mayer et al., 2020), on 24 selected KP strains representing both origins (12 human and 12 meat isolates) and taking into account STs and PFGE-macrorestriction profiles. Biofilm formation was also assessed in LB or LB+G with culture medium exchange after 12 h to promote biofilm growth. Additionally, biofilm cultures were incubated both under AE and μAE conditions since it has been reported that a μAE environment can promote biofilm formation by a bacterial pathogen in a lung-infection model (de la Fuente-Núñez et al., 2013).

The AA culture method allowed the growth of robust KP biofilms and proved to be more reproducible than the xCELLigence® system (Fig. 2A–B) for both, lower biofilm formers (ATCC 13883$^{\text{T}}$) and higher biofilm formers (KLEB-33). Biofilm formation by KP strains was highly dependent on the culture conditions, with a strain-specific response (Supplementary Fig. S3). The effect of glucose supplementation and/or microaerobiosis differed for each strain, with some strains showing opposite trends (Fig. 2). A higher number of statistically significant differences between strains was observed in LB+G μAE, indicating that this condition allows for greater discrimination between strains than the other culture conditions tested (Fig. 3). Although differences between the 4 culture conditions tested were not statistically significant, in average higher biofilm formation was observed in the LB+G μAE condition in both human and meat strains (Supplementary Fig. S4), and also in the ST307 and ST45 strains (Supplementary Fig. S5). Human isolates showed greater inter-strain variability than food-derived isolates, and formed in average more biofilm in all conditions tested (Supplementary Figs. S3 and S4), but no statistical significant differences were found compared with meat strains. No correlation between biofilm formation ability, origin and ST or PFGE molecular profiles could be observed in any of the tested conditions. However, a slight positive correlation was observed between biofilm formation and AMR profile for HMV+ strains ($R^2 = 0.64$, $p = 0.03$) under LB AE conditions.

As we did not observed any correlation with the origin and the biofilm formation regarding the cultivation methods mentioned above, we additionally studied a recently described flocculent phenotype denominated R-biofilm (Liu et al., 2020). This biofilm is thought to be generated as a protective response to stress generated by DNA damage due to double-strand breaks (DSBs). Some antibiotics such as quinolones used in clinical routine can cause DSBs by inhibiting the ligase domain of topoisomerases. In our 24 strains studied in biofilm formation experiments, all strains are resistant to the quinolones ciprofloxacin and nalidixic acid, so we wanted to know whether this resistance could correlate with the ability to form R biofilms, and whether this biofilm

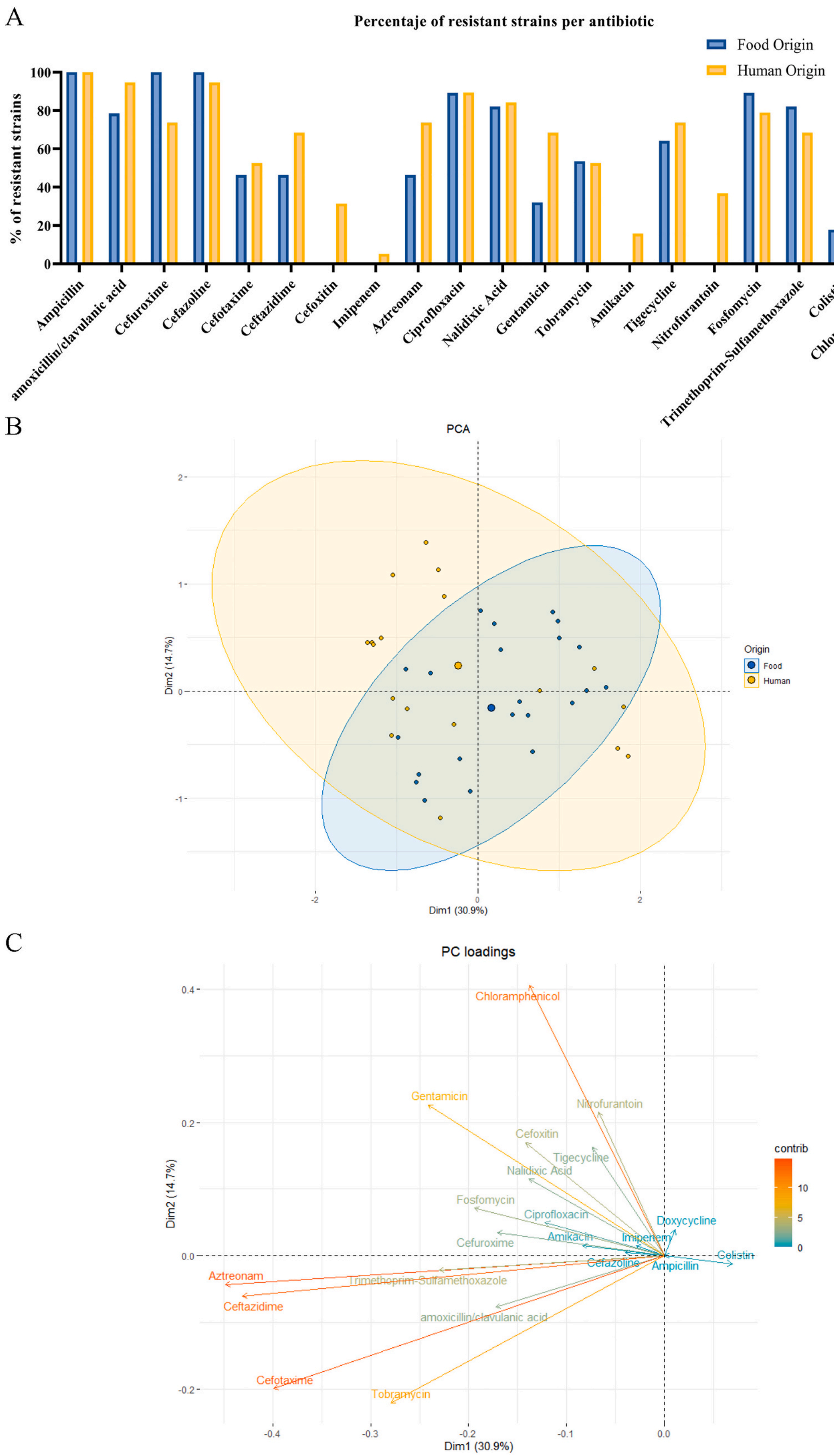

Fig. 1. Percentage of antibiotic resistant strains among human ($n = 19$) and food-borne ($n = 28$) strains isolated in this study (A). Principal component analysis (PCA) performed on the AMR profile antibiogram data showing that both populations overlap (B). Contribution of each antibiotic (discrimination capacity) to all strains studied, both human and food-borne (C).

Table 1
Hypermucoviscous phenotype (HMV) in 8 different culture conditions assessed by the string test (Shon et al., 2013). Only HMV+ results are shown.

		Culture conditions							
Strain	**Origin**	**LB+G 0,4% µAE**	**LB+G 0,4% AE**	**LB µAE**	**LB AE**	**Columbia Agar, AE**	**Columbia Agar, µAE**	**Agar EMB Levine, AE**	**Agar EMB Levine, µAE**
8-KLEB	Food	-	-	+(>5mm)	-	-	-	-	-
15-KLEB		-	-	±(≈5mm)	-	-	-	-	-
21-KLEB		++(>10mm)	++(>10mm)	+(>5mm)	+(>5mm)	+(>5mm)	++(>10mm)	++(>10mm)	++(>10mm)
22-KLEB		++(>10mm)	++(>10mm)	+(>5mm)	+(>5mm)	+(>5mm)	++(>10mm)	++(>10mm)	++(>10mm)
26-KLEB		-	+(>5mm)	-	-	+(>5mm)	-	-	-
33-KLEB	Human	±(≈5mm)	-	-	-	-	-	-	-
42-KLEB		±(≈5mm)	-	-	-	-	-	-	-

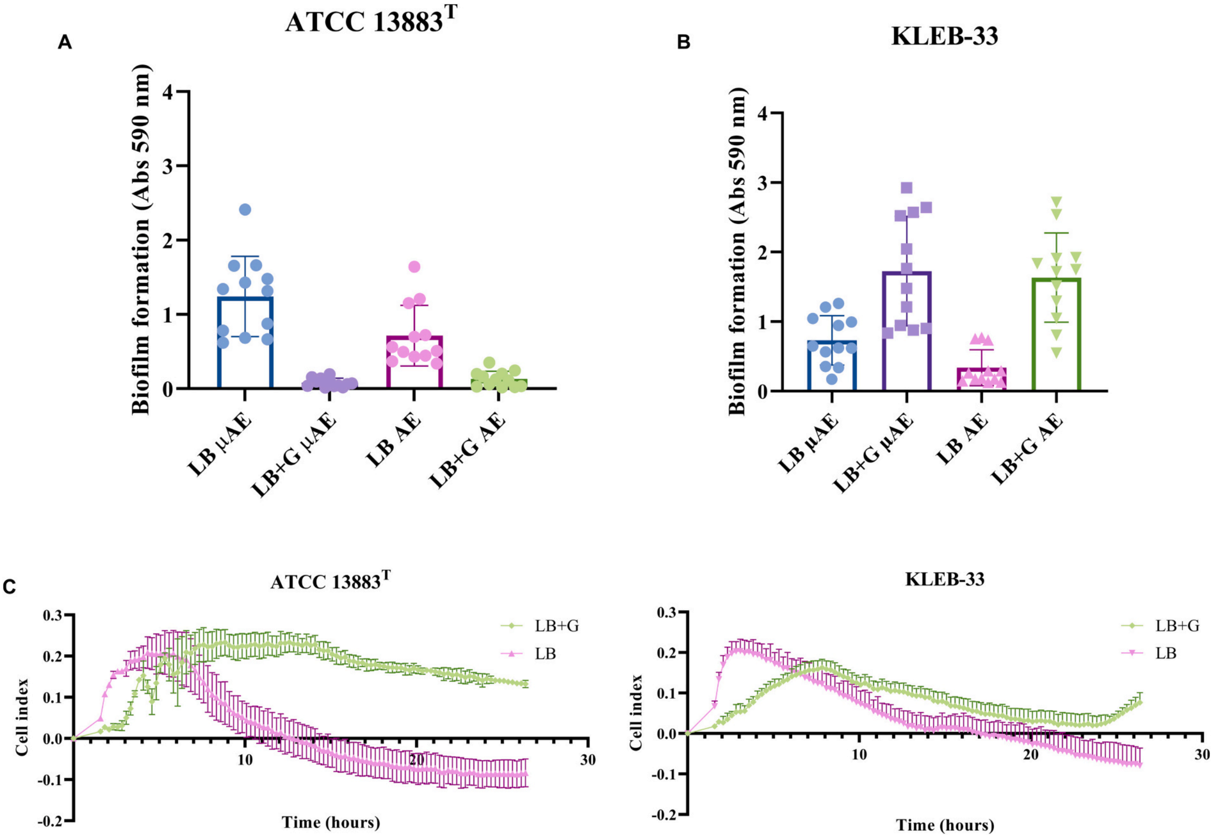

Fig. 2. Biofilm formation by KP type strain (ATCC 13883^T) (A) and MDR clinical isolate KLEB-33 (B) in the active attachment biofilm culture method under 4 different culture conditions: LB culture medium (LB) and LB supplemented with 0.4 % glucose (LB+G) in aerobiosis (AE) or microaerobiosis (µAE). Biofilms were stained and quantified using CV. Values represented are from 4 independent experiments with 3 replicates each. (C) Biofilm formation by the same strains measured with the xCELLigence® real-time biofilm formation monitoring system.

type could correlate with origin, as strains of human origin are expected to be exposed to these antibiotics more frequently than strains of meat origin. The addition of bleomycin induced the formation of R-biofilms in 7 out of the 24 strains tested (Fig. 5) both of food (KLEB-11, KLEB-21, KLEB-28) and human origin (KLEB-33, KLEB-35, KLEB-42 and KLEB-45a). We did not observe any correlation between the ability to form biofilm R with origin or with ST. However, we observed some prevalence of R biofilm formation among strains from ST326 (2) and ST15 (3) (Fig. 3). R-biofilm formation was inhibited by DNase I in 4 of the 7 biofilm R-forming strains, indicating that DNA is a key component of these flocculent ring-shaped deposits in KP strains. DNase I did not produce R-biofilm-like structures in media without bacteria and growth of KP strains was not affected by this enzyme (Supplementary Fig. S6).

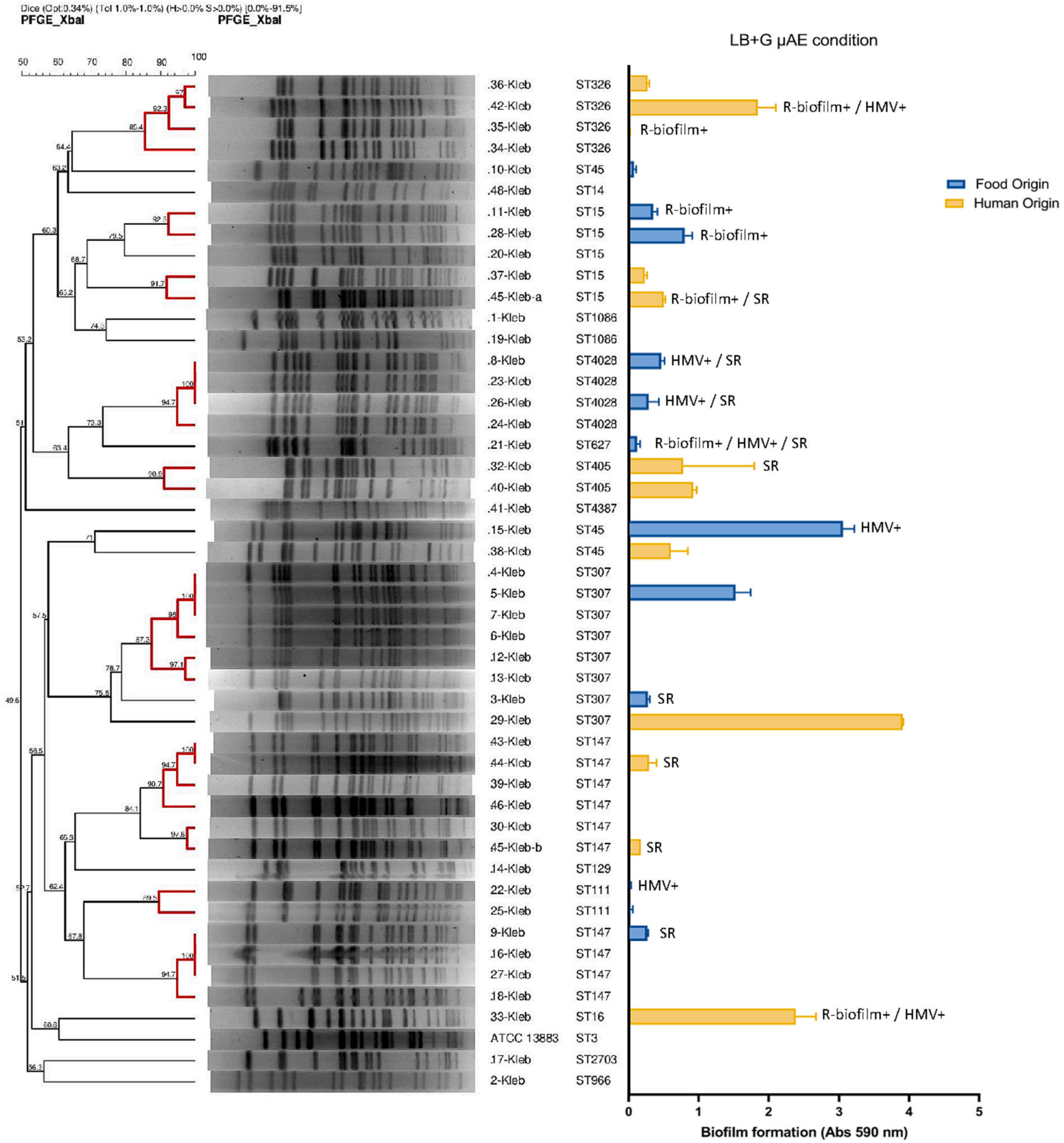

Fig. 3. Biofilm formation capacity of the in AA model quantified by CV staining in LB medium supplemented with 0.4 % glucose (LB+G) in microaerobiosis (μAE) for the 12 food (blue bars) and 12 human (yellow bars) KP strains and the type strain ATCC 13883^T. Strains were ordered according to PFGE analysis (left side) and ST data. HMV+, R biofilm-forming and serum-resistant (SR) strains are also indicated. Biofilm formation under LB AE, LB μAE and LB+G AE conditions is shown in Supplementary Fig. S3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Serum resistance of meat and human-origin strains

Capsule hyperproduction, a highly important virulence factor, has been correlated to trigger serum resistance in clinical infections (Guerra et al., 2022). We studied this phenotype to assess the correlation with biofilm formation and similarities between our two populations. In our experiments, only 8 of the 24 strains tested showed resistance to human serum (Fig. 4A) after growth under the biofilm promoting conditions selected previously (LB+G μAE) (Fig. 3), including 4 strains of meat origin (KLEB-3, KLEB-8, KLEB-9, KLEB-21) and 4 strains of human origin (KLEB-32, KLEB-44, KLEB-45a, KLEB-45b). Hence, no significant differences in sensitivity to human serum were found depending on strain origin. Moreover, no correlation was observed between serum resistance and biofilm formation or HMV phenotype under the conditions tested.

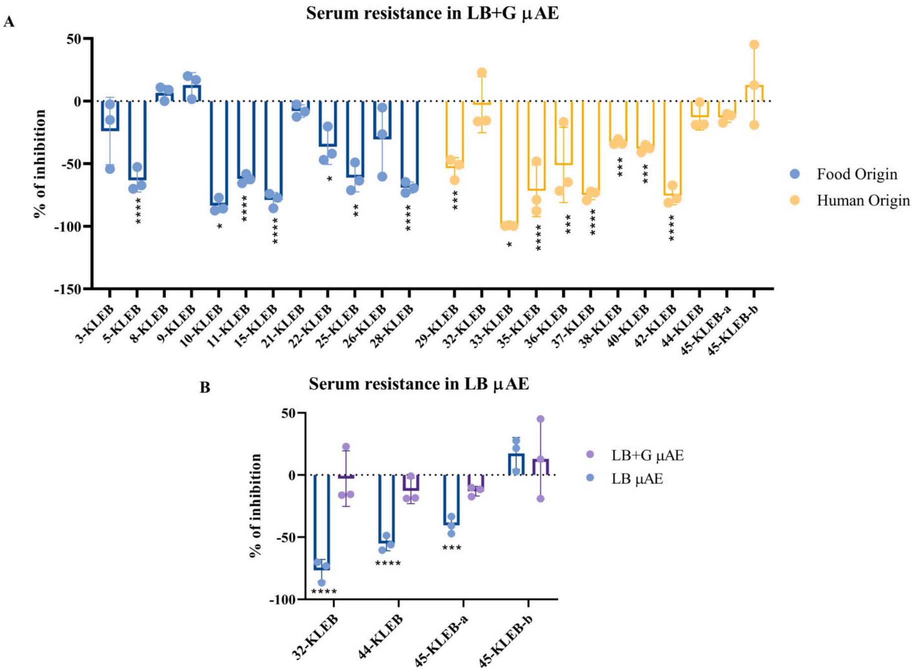

Fig. 4. Resistance to human serum, quantified as CFU/mL and expressed as % inhibition compared to control without serum treatment (in PBS) for 24 strains of KP of food (blue bars) and human (yellow bars) origin (A). Bacterial inoculum was incubated 24 h under LB+G µAE (A) or LB µAE (B) conditions. 3 experimental replicates were performed for each strain and in control and treatment samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Since serum resistance might depend on the presence of capsule, which has been described to be stimulated by the presence of glucose (Chen et al., 2020), we tested the effect of glucose depletion on serum sensitivity in the 4 resistant strains of human origin, which have different behaviour in biofilm formation (AA method) with or without glucose supplementation. We observed that in 3 of them the absence of carbohydrates significantly increased the sensitivity to human serum (Fig. 4B).

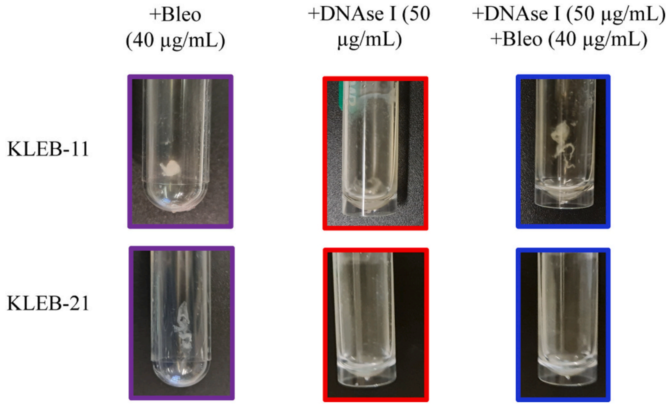

Fig. 5. Representative images of R-biofilms showing different response to DNase treatment in 2 KP strains. A strain was considered positive when a ring shaped (or similar) aggregate was visible after growth in LB supplemented with the antibiotic Bleomycin and this deposit was able to withstand a tube tipping without rupturing. The effect of DNase I alone (red) and in combination with bleomycin (blue) was also studied to assess whether DNA was a structural component of R biofilms, as previously described in the literature (Liu et al., 2020). The effect on growth was also studied (Supplementary Fig. S6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In this study, we aimed to compare MDR strains of KP of food and clinical origin in terms of pathogenic traits, biofilm capacity and AMR profile to investigate possible foodborne transmission risk. Furthermore, we aimed to establish a standardised methodology for biofilm formation quantification and assess some of the most relevant virulence traits used to evaluate the pathogenic potential of strains in vitro.

Díaz-Jiménez et al. (2020) had already demonstrated consumer exposure through poultry meat to *Enterobacteriaceae* with the capacity to develop severe extraintestinal infections due to bacterial virulence traits and/or antibiotic resistance. Of the 100 meat samples analysed, we recovered 28 strains ESBL-producing of KP, which were used in the present study. Eight of the 11 STs identified among the 28 foodborne KPs had been previously reported in human clinical isolates, including recognised high-risk clones such as ST15, ST307 or ST147 (Peirano et al., 2020). Several studies have shown that these STs are among those associated with high rates of AMR, including MDR and extensively drug resistant strains (XDR) of KP globally (Ferreira Raro et al., 2023; Navon-Venezia et al., 2017). Notably, ST15, ST307 and ST147 isolates accounted for 50 % of our food strains. We also found in our study that turkey meat was significantly more contaminated with ESBL-producing strains than chicken (26 and 2 strains isolated, respectively). A significantly higher level of contamination with MDR *E. coli* in turkey meat in comparison with chicken samples was also found in previous studies (Davis et al., 2018; Díaz-Jiménez et al., 2020). The differences found for turkey meat may be related to a longer exposure to antibiotics due to the much longer fattening period.

The global spread of successful clonal lineages of KP is associated with the spread of ESBLs and carbapenemases, which poses an extreme therapeutic challenge. Therefore, it is crucial to follow their dissemination to prevent further spread. In our study, all foodborne strains were MDR except KLEB-41, which was notably the only clone of ST4387, an ST not previously reported to be high risk in this species. Therefore, it seems clear that the presence of these MDR clones in poultry meat means that there is a real risk of foodborne transmission, as recently reported by Scientific Committee of the Spanish Agency for Food Safety and Nutrition on the survey of biological hazards of food safety concern in Spain (Franco Abuín et al., 2023).

For comparative purposes, 19 clinical MDR strains were included in this study. The PFGE analysis showed heterogeneity within the strains belonging to the same STs. We have compared the AMR profiles of both populations and important virulence factors, such as HMV, biofilm, and human-complement resistance, as these are some of the main factors determining the progression and severity of infection (González-Ferrer et al., 2021). PCA analysis showed indistinguishable populations in terms of AMR profile, although no food-resistant strains were found for cefoxitin, imipenem, amikacin, nitrofurantoin. The most common fully identical AMR profiles (present in 60 % of the strains) were ampicillin, amoxicillin, cefuroxime, cefazoline, ciprofloxacin, nalidixic acid and fosfomycin, and doxycycline, present in 13 human and 16 food-origin strains. In terms of virulence factors, the HMV phenotype was found in 7 of the 48 strains studied. Of these, 5 strains were of food origin and 2 were of human origin, suggesting that this virulence factor may indicate differences in the two populations, although the limited number of HMV+ strains obtained makes it difficult to draw conclusions and points to the need for further studies. However, the high percentage of HMV strains recovered from food samples in other studies (Yang et al., 2019) may indicate that this characteristic is not a marker of hypervirulence in all cases (Catalán-Nájera et al., 2017; Walker and Miller, 2020).

We also observed that the HMV phenotype was strongly influenced by culture conditions and found no association with strain ST and biofilm formation capacity, although the HMV phenotype and biofilm hyperformation have been reported to be very common in bacteremic strains of KP (González-Ferrer et al., 2021). This dependence on culture medium might have been the cause of the failure to identify the HMV phenotype among 56 *Klebsiella* spp. isolates from commensal, community-acquired and nosocomial infection strains, as reported in a recent study (Gómez et al., 2021). In contrast, here we found a positive correlation in HMV+ strains between biofilm formation and AMR profile, suggesting that testing alternative culture conditions could allow the detection of more HMV strains. However, this correlation was not observed in the best discriminating condition for biofilm formation (LB+G μAE), so further studies are needed to draw conclusions on the correlation of HMV phenotype and biofilm formation. In agreement with previous studies (Walker and Miller, 2020), our results showed that HMV and serum resistance are separate traits in KP. However, the higher serum sensitivity observed in our strains, probably related to lower capsule production, might be caused by low glucose concentration used in our study, as different concentrations might reach different conclusions (Walker and Miller, 2020).

Biofilm formation is crucial for the persistence of KP in the environment and during infection (Guerra et al., 2022). Furthermore, biofilm formation is also a key factor involved in the persistence of KP MDR species on food industry surfaces (González-Rivas et al., 2018) and should therefore be considered a crucial feature to characterise the pathogenic potential of strains. Discrepancies found in the literature on biofilm formation and antibiotic resistance profile in KP (Cusumano et al., 2019; Ramos-Vivas et al., 2019) prompted us to optimise the methodology applied to assess the biofilm formation capacity of selected KP strains. Although the xCELLigence® system that measures biofilm formation in real time with high sensitivity, measuring both matrix and cell growth (total biofilm mass), and has been successfully applied to assess monospecific and multispecific biofilm formation (Muras et al., 2018), it did not produce reproducible biofilm communities in the KP strains analysed in this study. Compared with previously reported CV staining procedures of biofilms formed at the bottom of wells in microtiter plates and the xCELLigence® system, the KP strains used in this study had substantially more repeatability with the active attachment (AA) system. This method has been already reported to produce robust and repeatable results for certain ESKAPE species (Mayer et al., 2020). The AA biofilm cultivation method could reproducibly detect differences in the response to culture conditions, even in lower biofilm formers such as KP ATCC 13883^{T} (Fig. 2A). Strong differences in biofilm formation ability were observed between strains, which were also strongly affected by culture conditions, indicating the importance of standardisation of biofilm methodologies to assess this important virulence-associated trait.

Despite studies describing a link between AMR and biofilm formation in KP (Cusumano et al., 2019; Ramos-Vivas et al., 2019), no association between these traits was found in this study. Similarly, no correlation was found between the biofilm formation capacity of the strains and their origin, HMV phenotype, PFGE, or ST profile, in any of the conditions tested. Furthermore, although some authors have reported that glucose supplementation leads to inhibition of biofilm in KP (Chen et al., 2020), we have not observed a common behavioural pattern in the response of the KP strains used to the presence of glucose in the medium (Supplementary Fig. S3). In some cases, a completely opposite biofilm-forming response to glucose addition was observed (Fig. 2). Strains of human origin showed a higher inter-strain variability in biofilm levels than those of food origin and this result could be related to the fact that they come from many different clinical sources, in contrast to strains of food origin, which are mostly derived from turkey meat. We found that LB supplemented with glucose in microaerobiosis was the best culture conditions for strain discrimination based on biofilm formation. Interestingly, ST307 strains were highly biofilm-forming in this condition, a ST recently correlated with high-risk clones (Peirano et al., 2020). Moreover, in this condition, strains formed on average more biofilm in both food and human strains. It should be noted that glucose availability and microaerobiosis conditions could be common in food processing environments, promoting the persistence of biofilm forming strains on food industry surfaces. The effect of this condition could perhaps be explained if low oxygen availability could promote biofilm formation and matrix production, which could also be enhanced by glucose availability. Some authors have reported that low oxygen availability favours biofilm dispersal and colonisation of new surfaces in *P. aeruginosa* (An et al., 2010). In addition, it should also be noted that KP metabolises glucose by the butanediol fermentation pathway under conditions of low oxygen availability (i.e. microaerobiosis). Interestingly, exogenous addition of this compound was also associated with greatly increased virulence and biofilm formation in *P. aeruginosa* cystic fibrosis-related infections (Venkataraman et al., 2014). Therefore, high glucose availability under microaerobiosis could lead to higher 2,3-butanediol concentrations, resulting in enhanced biofilm formation and explaining our observations.

Additionally, a recently described new type of resistant biofilm form called R-biofilm (Liu et al., 2020) was assessed in our strains. We observed R-biofilms in 7 out of 24 strains (29.16 %) (3 food-borne and 4 clinical), again indicating a homogeneity between food-borne and human strains in terms of the ability to form R-biofilms. It should be noted that this ability might be concentration-dependent, as all strains tested are MDR, and only 4 strains were completely inhibited by bleomycin (KLEB-9, KLEB-26, KLEB-29 and KLEB-40, data not shown). Of the 24 strains studied, 3 of the 4 ST15 strains were R-biofilm+, suggesting that this biofilm type could play an important role in persistence and resistance to antibiotic-mediated mechanisms, as ST15 is associated with high-risk strains disseminated worldwide (Peirano et al., 2020), but further studies are needed to corroborate this hypothesis. Although proteins have been described as the main component of matrix or R-biofilms (Liu et al., 2020), extracellular DNA was probably the main component of the R-biofilm in 4 of these strains. The importance of eDNA in biofilm formation has also been reported for other ESKAPE pathogens (Mayer et al., 2020) and is particularly relevant, as it can be considered a dissemination pathway for antibiotic-resistance genes (Oliveira et al., 2020).

5. Conclusion

In our study, we found no statistically significant differences in the AMR profile between some KP strains of food and human origin, suggesting that horizontal gene transfer could be playing a crucial role in the transmission of resistance between both ecosystem niches and the potential zoonotic transmission of pathogenic strains. In our conditions and strains tested, no link was found between strain origin, ST, HMV, or serum resistance with biofilm formation. However, we intend to make a methodology proposal regarding the detection of HMV using different culture conditions, and the analysis of biofilms in KP strains, as our experiments showed high reproducibility. The only clinically relevant difference we observed between food-borne and clinical KP populations was a slightly higher biofilm formation on average for strains of human origin, but without statistical significance. Our findings suggest that the same high risk of zoonosis from potentially virulent foodborne strains previously observed in *E. coli* is also present in other high-priority pathogens such as KP. This study further confirms that foodborne isolates of KP pose a risk to consumers and therefore this pathogen should be included in the surveillance of foodborne pathogens with a problematic risk of MDR infections and therapeutic failure.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110605.

Funding

The experiments performed in this work were financed with the projects and funds PID2019-104439RB-C21/AEI/10.13039/501100011033 (Agencia Estatal de Investigación, Spain); ED431B 2020/13 and ED431C 2021/11 (Xunta de Galicia); ED431E 2018/09 (BioReDes); GRUPIN IDI/2022/000033 by the Regional Ministry of Science of Asturias (IDI/2022/000033). Sergio Silva-Bea was supported with the “Formación de Profesorado Universitario” program (FPU21/01147). Manuel Romero was supported by the Maria Zambrano program from the Spanish Ministry of Universities.

CRediT authorship contribution statement

Sergio Silva-Bea: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft. **Manuel Romero:** Writing – review & editing. **Ana Parga:** Conceptualization, Investigation, Methodology. **Javier Fernández:** Resources. **Azucena Mora:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Ana Otero:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

References

Abreu-Salinas, F., Díaz-Jiménez, D., García-Meniño, I., Lumbreras, P., López-Beceiro, A. M., Fidalgo, L.E., Rodicio, M.R., Mora, A., Fernández, J., 2020. High prevalence and diversity of cephalosporin-resistant Enterobacteriaceae including Extraintestinal Pathogenic *E. coli* CC648 lineage in rural and urban dogs in Northwest Spain. Antibiotics 9 (8), 468. https://doi.org/10.3390/antibiotics9080468.

An, S., Wu, J., Zhang, L.-H., 2010. Modulation of *Pseudomonas aeruginosa* biofilm dispersal by a cyclic-Di-GMP phosphodiesterase with a putative hypoxia-sensing domain. Appl. Environ. Microbiol. 76 (24), 8160–8173. https://doi.org/10.1128/AEM.01233-10.

Aslam, B., Chaudhry, T.H., Arshad, M.I., Muzammil, S., Siddique, A.B., Yasmeen, N., Khurshid, M., Amir, A., Salman, M., Rasool, M.H., Xia, X., Baloch, Z., 2022. Distribution and genetic diversity of multi-drug-resistant *Klebsiella pneumoniae* at the human–animal–environment interface in Pakistan. Front. Microbiol. 13, 898248 https://doi.org/10.3389/fmicb.2022.898248.

Catalán-Nájera, J.C., Garza-Ramos, U., Barrios-Camacho, H., 2017. Hypervirulence and hypermucoviscosity: two different but complementary *Klebsiella* spp. phenotypes? Virulence 8 (7), 1111–1123. https://doi.org/10.1080/21505594.2017.1317412.

Chen, L., Wilksch, J.J., Liu, H., Zhang, X., Torres, V.V.L., Bi, W., Mandela, E., Cao, J., Li, J., Lithgow, T., Zhou, T., 2020. Investigation of LuxS-mediated quorum sensing in *Klebsiella pneumoniae*. J. Med. Microbiol. 69 (3), 402–413. https://doi.org/10.1099/jmm.0.001148.

Council of the European Union. (2023). Proposal for a COUNCIL RECOMMENDATION on stepping up EU actions to combat antimicrobial resistance in a one health approach. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32023H0622(01).

Cusumano, J.A., Caffrey, A.R., Daffinee, K.E., Luther, M.K., Lopes, V., LaPlante, K.L., 2019. Weak biofilm formation among carbapenem-resistant *Klebsiella pneumoniae*. Diagn. Microbiol. Infect. Dis. 95 (4), 114877 https://doi.org/10.1016/j.diagmicrobio.2019.114877.

Davis, G.S., Waits, K., Nordstrom, L., Grande, H., Weaver, B., Papp, K., Horwinski, J., Koch, B., Hungate, B.A., Liu, C.M., Price, L.B., 2018. Antibiotic-resistant *Escherichia coli* from retail poultry meat with different antibiotic use claims. BMC Microbiol. 18 (1), 174. https://doi.org/10.1186/s12866-018-1322-5.

de la Fuente-Núñez, C., Reffuveille, F., Fernández, L., Hancock, R.E., 2013. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Curr. Opin. Microbiol. 16 (5), 580–589. https://doi.org/10.1016/j.mib.2013.06.013.

Diancourt, L., Passet, V., Verhoef, J., Grimont, P.A.D., Brisse, S., 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J. Clin. Microbiol. 43 (8), 4178–4182. https://doi.org/10.1128/JCM.43.8.4178-4182.2005.

Díaz-Jiménez, D., García-Meniño, I., Fernández, J., García, V., Mora, A., 2020. Chicken and Turkey meat: consumer exposure to multidrug-resistant Enterobacteriaceae including mcr-carriers, uropathogenic *E. coli* and high-risk lineages such as ST131. Int. J. Food Microbiol. 331, 108750 https://doi.org/10.1016/j.ijfoodmicro.2020.108750.

ECDC, WHO, 2023. Antimicrobial resistance surveillance in Europe 2023. In: 2021 data. EU Publications. https://doi.org/10.2900/727139. ISBN: 978-92-9498-633-7.

EFSA & ECDC, 2023. The European Union one health 2022 Zoonoses report. EFSA J. 21 (12) https://doi.org/10.2903/j.efsa.2023.8442.

Ferreira Raro, O.H., Nordmann, P., Dominguez Pino, M., Findlay, J., Poirel, L., 2023. Emergence of Carbapenemase-producing Hypervirulent *Klebsiella pneumoniae* in Switzerland. Antimicrob. Agents Chemother., e01424-22 https://doi.org/10.1128/aac.01424-22.

Franco Abuín, C.M., Calleja, C.A., Escámez, P.F., Moreno, V., Arribas, Moragas, G.S., Díaz, A.V., 2023. Report of the scientific Committee of the Spanish Agency for food safety and nutrition (AESAN) on the prospection of biological hazards of interest in food safety in Spain (2). *Food risk assess*. Europe 1 (1). https://doi.org/10.2903/sp.efsa.2023.FR-0003.

García, V., Lestón, L., Parga, A., García-Meniño, I., Fernández, J., Otero, A., Olsen, J.E., Herrero-Fresno, A., Mora, A., 2023. Genomics, biofilm formation and infection of bladder epithelial cells in potentially uropathogenic *Escherichia coli* (UPEC) from animal sources and human urinary tract infections (UTIs) further support food-borne transmission. One Health 16, 100558. https://doi.org/10.1016/j.onehlt.2023.100558.

García-Meniño, I., Forcelledo, L., Rosete, Y., García-Prieto, E., Escudero, D., Fernández, J., 2021. Spread of OXA-48-producing *Klebsiella pneumoniae* among COVID-19-infected patients: the storm after the storm. J. Infect. Public Health 14 (1), 50–52. https://doi.org/10.1016/j.jiph.2020.11.001.

Gómez, M., Valverde, A., del Campo, R., Rodríguez, J.M., Maldonado-Barragán, A., 2021. Phenotypic and molecular characterization of commensal. Community-Acquired and Nosocomial Klebsiella spp. Microorganisms 9 (11), 2344. https://doi.org/10.3390/microorganisms9112344.

González-Ferrer, S., Peñaloza, H.F., Budnick, J.A., Bain, W.G., Nordstrom, H.R., Lee, J.S., Van Tyne, D., 2021. Finding order in the Chaos: outstanding questions in *Klebsiella pneumoniae* pathogenesis. Infect. Immun. 89 (4), e00693-20 https://doi.org/10.1128/IAI.00693-20.

González-Rivas, F., Ripolles-Avila, C., Fontecha-Umaña, F., Ríos-Castillo, A.G., Rodríguez-Jerez, J.J., 2018. Biofilms in the spotlight: detection, quantification, and removal methods. Compr. Rev. Food Sci. Food Saf. 17 (5), 1261–1276. https://doi.org/10.1111/1541-4337.12378.

Guerra, M.E.S., Destro, G., Vieira, B., Lima, A.S., Ferraz, L.F.C., Hakansson, A.P., Darrieux, M., Converso, T.R., 2022. *Klebsiella pneumoniae* biofilms and their role in disease pathogenesis. Front. Cell. Infect. Microbiol. 12, 877995 https://doi.org/10.3389/fcimb.2022.877995.

Herzberg, M., Kaye, I.K., Peti, W., Wood, T.K., 2006. YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. J. Bacteriol. 188 (2), 587–598. https://doi.org/10.1128/JB.188.2.587-598.2006.

Holt, K.E., Wertheim, H., Zadoks, R.N., Baker, S., Whitehouse, C.A., Dance, D., Jenney, A., Connor, T.R., Hsu, L.Y., Severin, J., Brisse, S., Cao, H., Wilksch, J., Gorrie, C., Schultz, M.B., Edwards, D.J., Nguyen, K.V., Nguyen, T.V., Dao, T.T., Thomson, N.R., 2015. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. Proc. Natl. Acad. Sci. 112 (27) https://doi.org/10.1073/pnas.1501049112.

Hu, Y., Anes, J., Devineau, S., Fanning, S., 2021. *Klebsiella pneumoniae*: prevalence, reservoirs, antimicrobial resistance, pathogenicity, and infection: A hitherto unrecognized zoonotic bacterium. Foodborne Pathog. Dis. 18 (2), 63–84. https://doi.org/10.1089/fpd.2020.2847.

Junka, A.F., Janczura, A., Smutnicka, D., Mączyńska, B., Secewicz, A., Nowicka, J., Bartoszewicz, M., Gościniak, G., 2012. Use of the real time xCelligence system for purposes of medical microbiology. Pol. J. Microbiol. 61 (3), 191–197. https://doi.org/10.33073/pjm-2012-024.

Liu, Y., Pan, C., Ye, L., Si, Y., Bi, C., Hua, X., Yu, Y., Zhu, L., Wang, H., 2020. Nonclassical biofilms induced by DNA breaks in *Klebsiella pneumoniae*. mSphere 5 (3), e00336-20. https://doi.org/10.1128/mSphere.00336-20.

Lv, J., Zhu, J., Wang, T., Xie, X., Wang, T., Zhu, Z., Chen, L., Zhong, F., Du, H., 2022. The role of the two-component QseBC signaling system in biofilm formation and virulence of Hypervirulent *Klebsiella pneumoniae* ATCC43816. Front. Microbiol. 13, 817494 https://doi.org/10.3389/fmicb.2022.817494.

Magiorakos, A.-P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18 (3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x.

Mayer, C., Muras, A., Parga, A., Romero, M., Rumbo-Feal, S., Poza, M., Ramos-Vivas, J., Otero, A., 2020. Quorum sensing as a target for controlling surface associated motility and biofilm formation in *Acinetobacter baumannii* ATCC® 17978TM. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.565548.

McLellan, J.E., Pitcher, J.I., Ballard, S.A., Grabsch, E.A., Bell, J.M., Barton, M., Grayson, M.L., 2018. Superbugs in the supermarket? Assessing the rate of contamination with third-generation cephalosporin-resistant gram-negative bacteria in fresh Australian pork and chicken. Antimicrobial Resistance & Infection Control 7 (1), 30. https://doi.org/10.1186/s13756-018-0322-4.

Muras, A., Mayer, C., Romero, M., Camino, T., Ferrer, M.D., Mira, A., Otero, A., 2018. Inhibition of *Streptococcus mutans* biofilm formation by extracts of *Tenacibaculum* sp. 20J, a bacterium with wide-spectrum quorum quenching activity. *Journal of Oral*. Microbiology 10 (1), 1429788. https://doi.org/10.1080/20002297.2018.1429788.

Muras, A., Otero-Casal, P., Blanc, V., Otero, A., 2020. Acyl homoserine lactone-mediated quorum sensing in the oral cavity: A paradigm revisited. Sci. Rep. 10 (1), 9800. https://doi.org/10.1038/s41598-020-66704-4.

Murray, C.J., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S.C., Browne, A.J., Chipeta, M.G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B.H., Kumaran, E.A.P., McManigal, B., Naghavi, M., 2022. Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. Lancet 399 (10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0.

Navon-Venezia, S., Kondratyeva, K., Carattoli, A., 2017. *Klebsiella pneumoniae*: A major worldwide source and shuttle for antibiotic resistance. FEMS Microbiol. Rev. 41 (3), 252–275. https://doi.org/10.1093/femsre/fux013.

Oliveira, M., Nunes, M., Barreto Crespo, M.T., Silva, A.F., 2020. The environmental contribution to the dissemination of carbapenem and (fluoro)quinolone resistance genes by discharged and reused wastewater effluents: the role of cellular and extracellular DNA. Water Res. 182, 116011 https://doi.org/10.1016/j.watres.2020.116011.

O'Neill, J., 2016. Tackling drug-resistant infections globally: final report and recommendations. Review on Antimicrobial Resistance. https://wellcomecollection.org/works/thvwsuba.

Padilla, E., Llobet, E., Doménech-Sánchez, A., Martínez-Martínez, L., Bengoechea, J.A., Albertí, S., 2010. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrob. Agents Chemother. 54 (1), 177–183. https://doi.org/10.1128/AAC.00715-09.

Parga, A., Muras, A., Otero-Casal, P., Arredondo, A., Soler-Ollé, A., Àlvarez, G., Alcaraz, L.D., Mira, A., Blanc, V., Otero, A., 2023. The quorum quenching enzyme Aii20J modifies in vitro periodontal biofilm formation. Front. Cell. Infect. Microbiol. 13, 1118630. https://doi.org/10.3389/fcimb.2023.1118630.

Patil, A., Banerji, R., Kanojiya, P., Saroj, S.D., 2021. Foodborne ESKAPE biofilms and antimicrobial resistance: lessons learned from clinical isolates. Pathogens and Global Health 115 (6), 339–356. https://doi.org/10.1080/20477724.2021.1916158.

Peirano, G., Chen, L., Kreiswirth, B.N., Pitout, J.D.D., 2020. Emerging antimicrobial-resistant high-risk *Klebsiella pneumoniae* clones ST307 and ST147. Antimicrob. Agents Chemother. 64 (10), e01148-20 https://doi.org/10.1128/AAC.01148-20.

Poirel, L., Walsh, T.R., Cuvillier, V., Nordmann, P., 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagn. Microbiol. Infect. Dis. 70 (1), 119–123. https://doi.org/10.1016/j.diagmicrobio.2010.12.002.

Ramos-Vivas, J., Chapartegui-González, I., Fernández-Martínez, M., González-Rico, C., Fortún, J., Escudero, R., Marco, F., Linares, L., Montejo, M., Aranzamendi, M., Muñoz, P., Valerio, M., Aguado, J.M., Resino, E., Ahufinger, I.G., Vega, A.P., Martínez-Martínez, L., Fariñas, M.C., 2019. Biofilm formation by multidrug resistant Enterobacteriaceae strains isolated from solid organ transplant recipients. Sci. Rep. 9 (1), 8928. https://doi.org/10.1038/s41598-019-45060-y.

Ribot, E. M., Fair, M. A., Gautom, R., Cameron, D. N., Hunter, S. B., Swaminathan, B., & Barrett, T. J. (2006). Standardization of Pulsed-Field Gel Electrophoresis Protocols for the Subtyping of *Escherichia coli* O157:H7, *Salmonella* , and *Shigella* for PulseNet. Foodborne Pathog. Dis., 3(1), 59–67. doi:https://doi.org/10.1089/fpd.2006.3.59.

Riley, L.W., 2020. Extraintestinal foodborne pathogens. Annu. Rev. Food Sci. Technol. 11 (1), 275–294. https://doi.org/10.1146/annurev-food-032519-051618.

Santaniello, A., Sansone, M., Fioretti, A., Menna, L.F., 2020. Systematic review and Meta-analysis of the occurrence of ESKAPE Bacteria Group in Dogs, and the related zoonotic risk in animal-assisted therapy, and in animal-assisted activity in the health context. Int. J. Environ. Res. Public Health 17 (9), 3278. https://doi.org/10.3390/ijerph17093278.

Sequeira, R.P., McDonald, J.A.K., Marchesi, J.R., Clarke, T.B., 2020. Commensal Bacteroidetes protect against *Klebsiella pneumoniae* colonization and transmission through IL-36 signalling. Nat. Microbiol. 5 (2), 304–313. https://doi.org/10.1038/s41564-019-0640-1.

Shon, A.S., Bajwa, R.P.S., Russo, T.A., 2013. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: A new and dangerous breed. Virulence 4 (2), 107–118. https://doi.org/10.4161/viru.22718.

Soto, E., LaMon, V., Griffin, M., Keirstead, N., Beierschmitt, A., Palmour, R., 2012. Phenotypic and genotypic characterization of *Klebsiella pneumoniae* isolates recovered form nonhuman primates. J. Wildl. Dis. 48 (3), 603–611. https://doi.org/10.7589/0090-3558-48.3.603.

Venkataraman, A., Rosenbaum, M.A., Werner, J.J., Winans, S.C., Angenent, L.T., 2014. Metabolite transfer with the fermentation product 2,3-butanediol enhances virulence by *Pseudomonas aeruginosa*. ISME J. 8 (6), 1210–1220. https://doi.org/10.1038/ismej.2013.232.

Walker, K.A., Miller, V.L., 2020. The intersection of capsule gene expression, hypermucoviscosity and hypervirulence in *Klebsiella pneumoniae*. Curr. Opin. Microbiol. 54, 95–102. https://doi.org/10.1016/j.mib.2020.01.006.

Wareth, G., Neubauer, H., 2021. The animal-foods-environment interface of *Klebsiella pneumoniae* in Germany: An observational study on pathogenicity, resistance development and the current situation. Vet. Res. 52 (1), 16. https://doi.org/10.1186/s13567-020-00875-w.

WHO. (2017). *Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis*. Geneva: World Health Organization; (WHO/EMP/IAU/2017.12). https://www.who.int/publications-detail-redirect/WHO-EMP-IAU-2017.12.

WHO, 2023. *GLASS manual for antimicrobial resistance surveillance in common Bacteria causing human infection*. World Health. Organization. ISBN: 978-92-4-007660-0. https://www.who.int/publications/i/item/9789240076600.

Wu, X., Liu, J., Feng, J., Shabbir, M.A.B., Feng, Y., Guo, R., Zhou, M., Hou, S., Wang, G., Hao, H., Cheng, G., Wang, Y., 2022. Epidemiology, environmental risks, virulence, and resistance determinants of *Klebsiella pneumoniae* from dairy cows in Hubei. China. Frontiers in Microbiology 13, 858799. https://doi.org/10.3389/fmicb.2022.858799.

Yang, F., Deng, B., Liao, W., Wang, P., Chen, P., Wei, J., 2019. High rate of multiresistant *Klebsiella pneumoniae* from human and animal origin. Infection and Drug Resistance 12, 2729–2737. https://doi.org/10.2147/IDR.S219155.

Zhang, S., Yang, G., Ye, Q., Wu, Q., Zhang, J., Huang, Y., 2018. Phenotypic and genotypic characterization of *Klebsiella pneumoniae* isolated from retail foods in China. Front. Microbiol. 9, 289. https://doi.org/10.3389/fmicb.2018.00289.