Shedding light on *Klebsiella pneumoniae* and *Escherichia coli* biofilms: from characterisation to new treatments

Victoria Ballén Torres

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Faculty of Medicine and Health Sciences

Department of Clinical Foundations

Doctoral program in Medicine and Translational Research

Shedding light on *Klebsiella pneumoniae* and *Escherichia coli* biofilms: from characterisation to new treatments

Doctoral thesis report submitted by Victoria Ballén Torres to obtain a Doctoral degree from the University of Barcelona

Thesis supervisor: Dr. Sara María Soto González

Department of Clinical Foundations

Barcelona Institute for Global Health- Barcelona University – Hospital Clínic of Barcelona

Barcelona, March 2022

La Dra. SARA Mª SOTO GONZALEZ, Associate Research Professor del Instituto de Salud Global de Barcelona (ISGlobal) y Profesora Asociada del Departamento de Fundamentos Clínicos de la Universitat de Barcelona.

CERTIFICA:

Que el trabajo de investigación titulado "Shedding light on *Klebsiella pneumoniae* and *Escherichia coli* biofilms: from characterisation to new treatments", presentado por VICTORIA DEL PILAR BALLÉN TORRES, se ha realizado en el laboratorio de microbiología de ISGlobal bajo mi dirección, y cumple todos los requisitos necesarios para su tramitación y posterior defensa ante el tribunal correspondiente.

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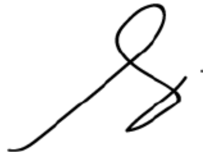

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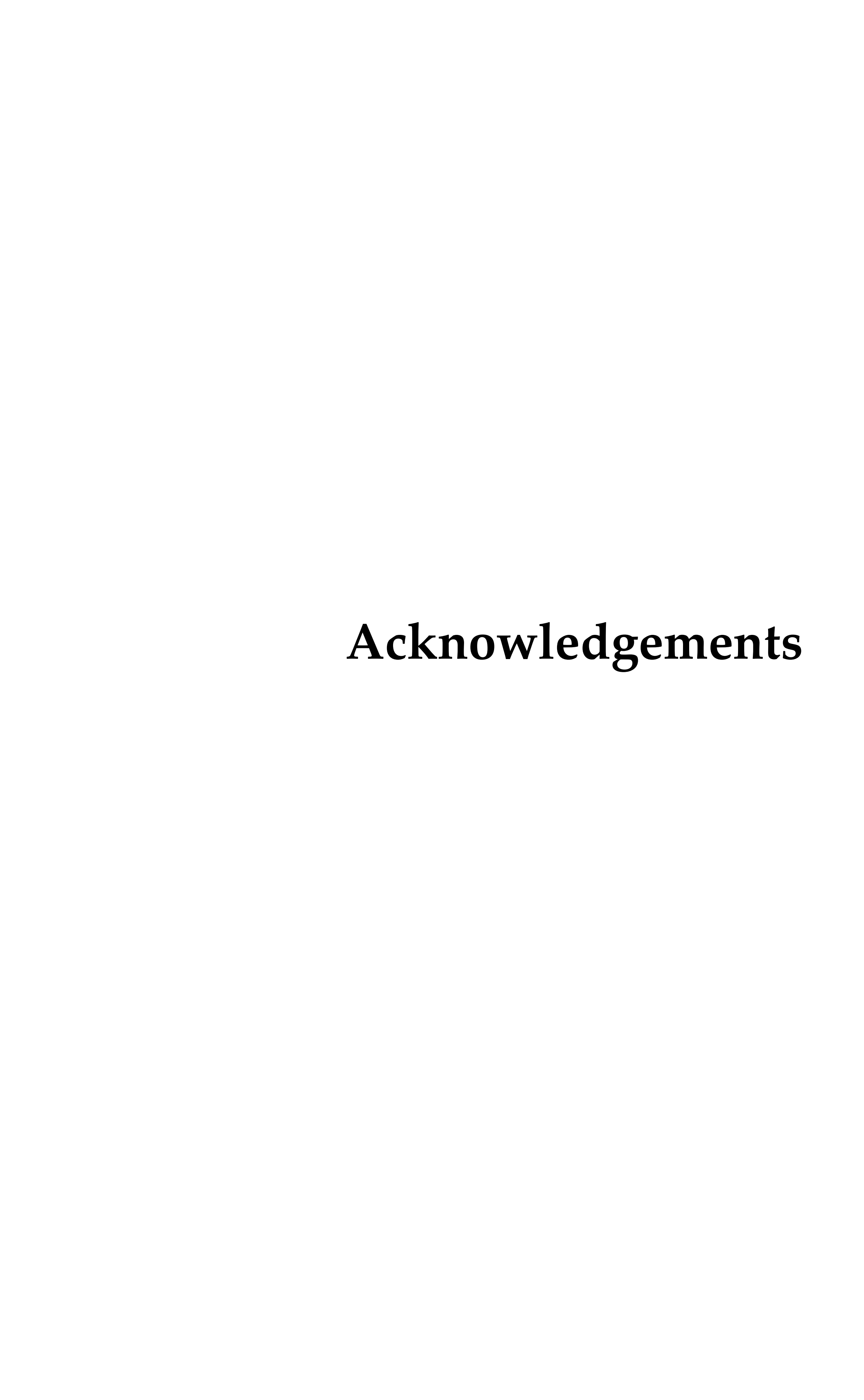

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I. Table of contents

I. Table of contents .... **I**

II. List of tables and figures .... **IX**

III. List of abbreviations .... **xiii**

IV. List of articles that comprise the thesis .... **XVII**

V. Thesis summary **1**

1. Características e importancia de las biopelículas 3

2. Biopelículas polimicrobianas 5

3. Dianas y moléculas para controlar las biopelículas 5

4. Determinantes de virulencia 8

5. Terapia antimicrobiana y resistencia a los antibióticos 11

6. Justificación de la tesis, hipótesis y objetivos 14

7. Resultados y discusión 16

8. Conclusiones 23

VI. Introduction **27**

1. Characteristics and relevance of biofilms 29

1.1. Biofilms definition 29

1.2. Stages of biofilm formation 29

1.2.1. Adhesion 29

1.2.2. Maturation 31

1.2.3. Dispersal process 33

1.3. Biofilms and their role in pathogenesis 34

1.3.1. Ventilator-associated pneumonia (VAP)..36

1.3.2. Bloodstream infections (BSIs) associated with intravenous catheters..37

1.3.3. Catheter-associated urinary tract infections (CAUTIs) 38

1.4. Immune evasion and antibiotic tolerance in biofilms...................... 38

1.4.1. Low antimicrobial penetration 39

1.4.2. Reduced growth rates and stress responses 40

1.4.3. Persister cells 40

1.4.4. Efflux pumps 41

1.4.5. Horizontal gene transfer (HGT) 42

2. Polymicrobial biofilms 43

2.1. Interspecies interactions.. 43

2.1.1. Cooperation 43

2.1.2. Competition..45

2.2. Polymicrobial biofilms formed by *K. pneumoniae* 46

3. Targets and molecules to control biofilms... 48

3.1. Adhesion inhibition 49

3.1.1. Ethyl acetate fraction of *Cocculus trilobus* 49

3.1.2. Cranberry polyphenols...49

3.1.3. *Ginkgo biloba* extract 49

3.1.4. Phloretin...50

3.2. Quorum sensing (QS) inhibitors 50

3.2.1. Furanone 51

3.2.2. Quercetin 52

3.2.3. Curcumin 52

3.3. Degradation of extracellular polymeric substances 53

3.3.1. Glycoside hydrolases 53

3.3.2. Proteases 53

3.3.3. Phages 54

3.3.4. Myricetin 54

3.4. Biofilms dispersion 55

3.4.1. Sequestration molecules 55

3.4.2. Antimicrobial peptides 55

4. Virulence determinants 58

4.1. Virulence factors found in both *K. pneumoniae* and *E. coli* strains... 60

4.1.1. Type 1 fimbriae 60

4.1.2. Siderophores 60

4.1.2.1. Enterobactin 61

4.1.2.2. Aerobactin 61

4.1.2.3. Salmochelin 62

4.1.2.4. Yersiniabactin 62

4.1.3. Capsular polysaccharide (K antigen) 62

4.1.4. Lipopolysaccharide (LPS) (O antigen) 64

4.1.5. Toxins 65

4.1.5.1. Cytotoxic necrotising factor 1 (CNF1) 65

4.1.5.2. α-Haemolysin (HlyA) 66

4.1.5.3. Colibactin 66

4.2. Specific virulence factors of *K. pneumoniae* 67

4.2.1. Type 3 fimbriae 67

4.3. Specific virulence factors of *E. coli* 68

4.3.1. Curli fimbriae 68

4.3.2. S-fimbriae 68

4.3.3. P-fimbriae 69

4.3.4. Poly-β-(1,6)-N-acetylglucosamine (PNAG) 69

4.3.5. Flagella (H antigen) 70

5. Antimicrobial therapy and resistance 72

5.1. Aminoglycosides 74

5.2. Quinolones 75

5.3. Trimethoprim-sulfamethoxazole 77

5.4. Chloramphenicol 77

5.5. Fosfomycin 78

5.6. Colistin 78

5.7. β-Lactams 79

5.8. β-Lactamase inhibitors 83

6. Relationship between virulence and antimicrobial resistance 84

VII. Work justification, Hypotheses and Objectives **87**

VIII. Results **91**

Paper 1. *Enterococcus faecalis* inhibits *Klebsiella pneumoniae* growth in polymicrobial biofilms in a glucose-enriched medium 93

Paper 2. Transposon Insertion in the *purL* Gene Induces Biofilm Depletion in *Escherichia coli* ATCC 25922 113

Manuscript 1. Antimicrobial and antibiofilm activity of a fruit extract containing proteolytic enzymes 137

Paper 3. Antibiotic Resistance and Virulence Profiles of *Klebsiella pneumoniae* Strains Isolated from Different Clinical Sources 163

Paper 4. Correlation between Antimicrobial Resistance, Virulence Determinants and Biofilm Formation Ability among Extraintestinal Pathogenic *Escherichia coli* Strains Isolated in Catalonia, Spain 177

IX. Discussion **195**

X. Conclusions **215**

XI. Bibliography **219**

II. List of tables and figures

List of tables

Table 1. Targets and some molecules to control biofilms 57

Table 2. Nomenclature of clinically important β-lactamase enzymes……82

List of figures

Figure 1. Bacterial adhesion to a surface in motile species. 30

Figure 2. Biofilm maturation... 33

Figure 3. Dispersal process.. 34

Figure 4. Biofilm-related infections... 35

Figure 5. Mechanisms of antimicrobial tolerance in biofilms. 39

Figure 6. Superfamilies of efflux pumps found in bacteria......................... 42

Figure 7. Interspecies interactions in biofilms. 44

Figure 8. Phloretin structure.. 50

Figure 9. The mechanisms of QS inhibiting agents in controlling bacterial biofilm formation.. 51

Figure 10. Furanone structure 51

Figure 11. Quercetin structure 52

Figure 12. Curcumin structure 52

Figure 14. Papain structure 54

Figure 13. Actinidin structure 54

Figure 15. Myricetin structure.. 54

Figure 16. Effects of c-di-GMP on biofilm formation. 56

Figure 17. Major virulence factors characterised in *K. pneumoniae*. 58

Figure 18. Virulence factors characterised in *E. coli*. 59

Figure 19. Type 1 fimbriae. 60

Figure 20. Siderophores 61

Figure 21. Capsular polysaccharide (K antigen) 63

Figure 22. Lipopolysaccharide structure. 64

Figure 23. Organisation of the *pks* genomic island. 67

Figure 24. Type 3 fimbriae. 67

Figure 25. Curli fimbriae. 68

Figure 26. *sfa* operon 69

Figure 27. P-fimbriae. 69

Figure 28. Biosynthesis of PNAG 70

Figure 29. Flagellar structure 71

Figure 30. Antibiotic targets and antibiotic resistance mechanisms 73

Figure 31. Horizontal gene transfer mechanisms. 74

Figure 32. Mechanisms of quinolone action. 76

III. List of abbreviations

ABC	ATP-binding cassette family
AHLs	Acyl-homoserine lactones
AI-2	Autoinducer 2
AMP	Adenosine 5′-monophosphate
AMPs	Antimicrobial peptides
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
Bap	Biofilm-associated protein
BSIs	Bloodstream infections
c-di-GMP	Cellular bis-(3′-5′)-cyclic dimeric guanosine monophosphate
CAUTIs	Catheter-associated urinary tract infections
CI	Competitive index
CNF1	Cytotoxic necrotising factor 1
CPS	Capsular polysaccharides
CR	Congo red
CRBSIs	Catheter-related bloodstream infections
DNA	Deoxyribonucleic acid
DspB	Dispersin B
eDNA	Extracellular deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
EARS-Net	European Antimicrobial Resistance Surveillance Network
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EPS	Extracellular polymeric substances
ESBLs	Extended-spectrum β-lactamases
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
GMP	Guanosine 5′-monophosphate
HGT	Horizontal gene transfer
HlyA	α-haemolysin
HMV	Hypermucoviscous
HPI	High-pathogenicity island

hvKp	Hypervirulent *K. pneumoniae*
ICU	Intensive care unit
IMP	Inosine monophosphate
Lcn2	Lipocalin-2
LPS	Lipopolysaccharide
MATE	Multidrug and toxic extrusion family
MBEC	Minimal biofilm eradication concentration
MBLs	Metallo-β-lactamases
MDR	Multidrug resistance
MFS	Major facilitator superfamily
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-susceptible *S. aureus*
OmpA	Outer membrane protein A
PBPs	Penicillin-binding proteins
PNAG	Poly-β-(1,6)-N-acetylglucosamine
QS	Quorum sensing
RNA	Ribonucleic acid
RND	Resistance nodulation-division family
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SMR	Small multidrug resistance family
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TSB	Trypticase soy broth
UTIs	Urinary tract infections
UPEC	Uropathogenic *E. coli*
VAP	Ventilator-associated pneumonia
VFGs	Virulence factor genes
WHO	World Health Organisation
Wt	Wild-type

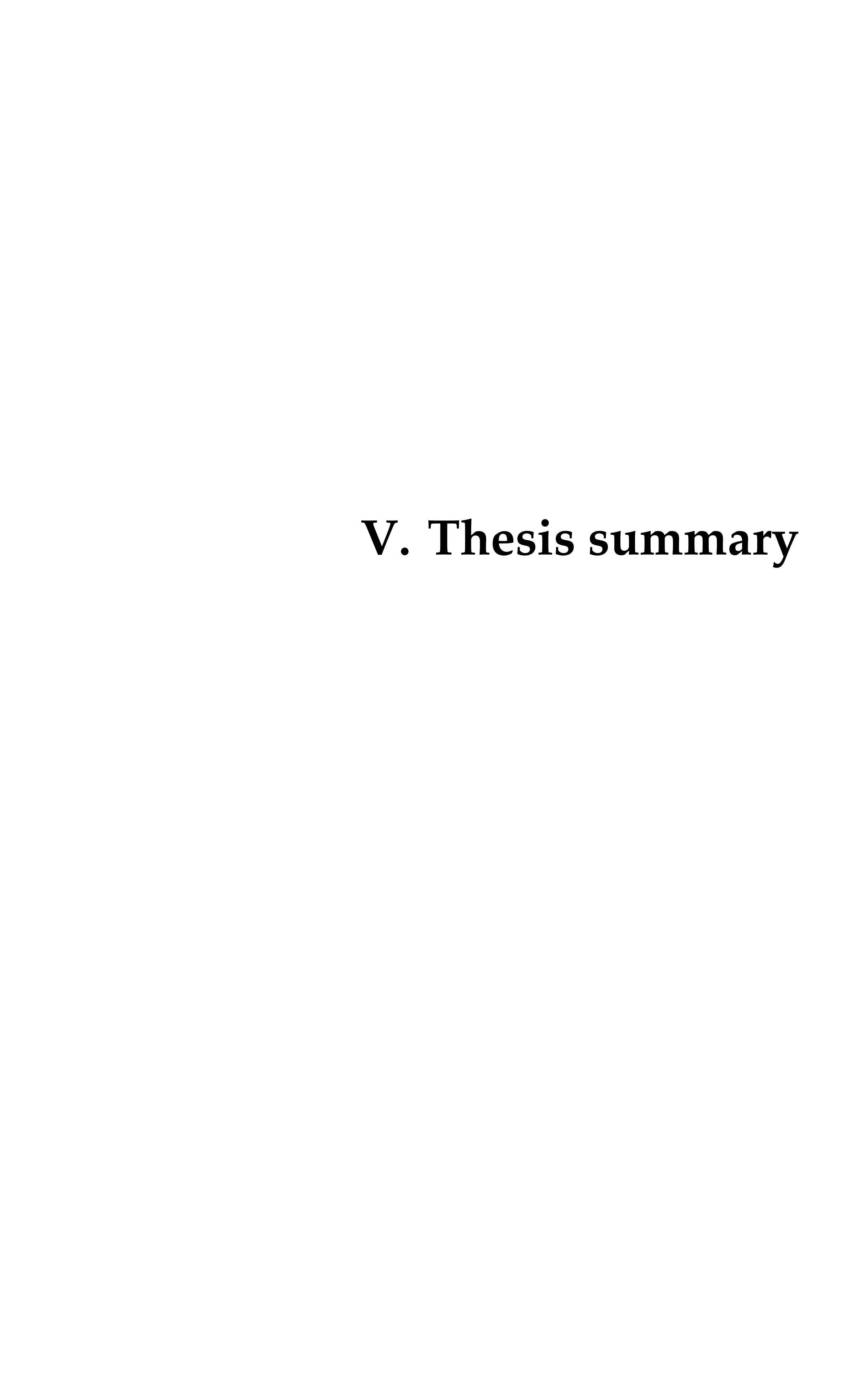
IV. List of articles that comprise the thesis

- **Ballén, V**., Ratia, C., Cepas, V., & Soto, S. M. (2020). Enterococcus faecalis inhibits *Klebsiella pneumoniae* growth in polymicrobial biofilms in a glucose-enriched medium. Biofouling, 36(7), 846–861. https://doi.org/10.1080/08927014.2020.1824272.

- Cepas, V., **Ballén, V**., Gabasa, Y., Ramírez, M., López, Y., & Soto, S. M. (2020). Transposon Insertion in the purL Gene Induces Biofilm Depletion in *Escherichia coli* ATCC 25922. Pathogens (Basel, Switzerland), 9(9), 774. https://doi.org/10.3390/pathogens9090774.

- **Ballén, V**., Gabasa, Y., Ratia, C., Ortega, R., Tejero, M., & Soto, S. (2021). Antibiotic Resistance and Virulence Profiles of *Klebsiella pneumoniae* Strains Isolated From Different Clinical Sources. Frontiers in cellular and infection microbiology, 11, 738223. https://doi.org/10.3389/fcimb.2021.738223.

- **Ballén, V**., Gabasa, Y., Ratia, C., Sánchez, M., & Soto, S. (2022). Correlation Between Antimicrobial Resistance, Virulence Determinants and Biofilm Formation Ability Among Extraintestinal Pathogenic *Escherichia coli* Strains Isolated in Catalonia, Spain. Frontiers in microbiology, 12, 803862. https://doi.org/10.3389/fmicb.2021.803862.



V. Thesis summary

1. Características e importancia de las biopelículas

Las biopelículas se han definido como agregados de células microbianas embebidas en una matriz de sustancias poliméricas extracelulares que ellas mismas producen. En la biopelícula, estas células pueden adherirse entre sí y a una amplia variedad de superficies bióticas o abióticas.

Esta tesis se centra principalmente en la formación de biopelículas formadas por *Klebsiella pneumoniae* y *Escherichia coli*, dos de los patógenos Gram negativos más importantes asociados a las infecciones hospitalarias y comunitarias.

El estilo de vida dentro de las biopelículas difiere considerablemente del que tienen las células bacterianas planctónicas, ya que las propiedades metabólicas dentro de las comunidades en las biopelículas son totalmente diferentes, lo que lleva a la evolución de una sofisticada arquitectura cuya formación implica un complejo proceso de desarrollo que tiene lugar en tres fases: adhesión, maduración y dispersión.

La adhesión se refiere a la primera interacción de las bacterias con una superficie. Las células segregan activamente sustancias químicas para adherirse reversiblemente, pero, posteriormente, la adhesión se convierte en permanente. Una vez que las células se han adherido de forma irreversible a la superficie, la biopelícula entra en la fase de maduración y se inicia la producción de la matriz, que permite la formación de comunidades más estructuradas que determinan la arquitectura final y la disposición espacial de la biopelícula. Esta estructura proporciona estabilidad mecánica, transporte de nutrientes y residuos, y protección contra la desecación, los agentes antimicrobianos, los anticuerpos y los efectos de la respuesta inmunitaria del huésped, incluida la acción del complemento y la fagocitosis. El proceso de dispersión es la etapa final del desarrollo de la biopelícula y puede darse mediante un proceso activo denominado dispersión o un proceso pasivo denominado desprendimiento. La dispersión es un proceso en el que las bacterias escapan de la biopelícula a través de la degradación

enzimática, dejando atrás las biopelículas erosionadas y permitiendo que las bacterias se dispersen a nuevos nichos. En cambio, en el desprendimiento, factores externos como la abrasión y las perturbaciones humanas actúan como inductores.

Se calcula que entre el 65% y el 80% de las infecciones humanas implican el desarrollo de biopelículas. Entre ellas, se encuentran las infecciones relacionadas con los tejidos, así como las infecciones asociadas a dispositivos permanentes. Las infecciones más comúnmente asociadas a las biopelículas están relacionadas con el uso de dispositivos invasivos, especialmente catéteres vasculares centrales, sondas urinarias y respiradores, que, respectivamente, conllevan al desarrollo de infecciones del torrente sanguíneo procedentes de catéteres venosos, infecciones del tracto urinario asociadas a catéter, y neumonías asociadas al ventilador.

Una de las mayores preocupaciones en el campo de la medicina es la dificultad de erradicar las biopelículas, ya que las bacterias presentes en ellas pueden evadir el sistema inmunitario al reducir la fagocitosis mediada por leucocitos y anticuerpos. Además, en comparación con sus análogas planctónicas, las biopelículas pueden ser hasta mil veces más tolerantes a los agentes antimicrobianos, lo que permite que las infecciones se vuelvan crónicas a pesar del tratamiento antibiótico. Aunque se han asociado varios mecanismos con la tolerancia a los antimicrobianos en las biopelículas, los más importantes incluyen: (1) Baja penetración de los antimicrobianos, donde la matriz actúa como una barrera física que limita o retrasa la penetración de los mismos; (2) Reducción de las tasas de crecimiento, ya que las bacterias de las capas más profundas tienen una menor actividad metabólica, una menor tasa de crecimiento y división, debido al gradiente de oxígeno y nutrientes, y de las respuestas al estrés; (3) Células persistentes, que se definen como variantes latentes de las células normales que se desarrollan en condiciones de estrés y muestran tasas de crecimiento cercanas a cero o extremadamente lentas, siendo muy tolerantes a los antibióticos; (4) Bombas de eflujo, proteínas de membrana responsables de la exportación de sustancias tóxicas, incluidos los antibióticos, desde el interior de la bacteria hacia el exterior; (5)

Transferencia horizontal de genes, que se da más fácilmente que en las bacterias planctónicas debido a la alta densidad de población existente en las biopelículas, lo que produce un aumento de las interacciones entre las células.

2. Biopelículas polimicrobianas

En la naturaleza, las biopelículas suelen estar formadas por varias especies que interactúan entre sí e influyen positiva o negativamente en el desarrollo de la comunidad. Las interacciones entre especies implican comunicación (normalmente a través del sistema de quorum sensing (QS)) y dan lugar a estrategias de cooperación o competencia metabólica entre los microorganismos presentes. En la cooperación o sinergia, las especies implicadas colaboran aumentando la formación de biopelículas y, posteriormente, en su tolerancia a los antibióticos y su virulencia. En el caso de la competencia o el antagonismo, la interacción resultante da una ventaja a una de las especies implicadas basada en la competencia de nutrientes o en la inhibición de sus homólogos. Sin embargo, en algunos casos, no se detecta ni sinergia ni competencia entre las especies implicadas, dando lugar a interacciones neutras.

En esta tesis, nos centraremos en las interacciones interespecie que se producen dentro de la biopelícula formada por *K. pneumoniae* y *E. faecalis*, dos uropatógenos de gran interés (Artículo 1).

3. Dianas y moléculas para controlar las biopelículas

El aumento de la resistencia a los antibióticos, combinado con la capacidad de algunas bacterias para formar biopelículas, es un reto para la salud pública y la investigación biomédica a nivel mundial. En muchos casos, la terapia antibiótica actual sólo puede reducir, pero no erradicar por completo las biopelículas, ya que las altas concentraciones de antibióticos necesarias para su completa erradicación pueden resultar tóxicas para el paciente. Por tanto, existe una necesidad urgente de desarrollar nuevas estrategias terapéuticas para contrarrestar el problema. Algunas de las estrategias esenciales para lograrlo son:

(i) Identificar nuevas dianas: Esta estrategia suele utilizar la inserción aleatoria de transposones, una herramienta de manipulación genética que permite mutaciones aleatorias a nivel del genoma bacteriano. Esta herramienta crea bibliotecas de mutantes en las que se identifican los factores genéticos responsables de la expresión de diferentes fenotipos.

En esta tesis se descubrió un nuevo gen implicado en la formación de biopelículas en *E. coli* mediante el uso de esta técnica (Artículo 2).

(ii) Diseño y cribado de nuevas moléculas: Actualmente se están investigando diferentes moléculas que intentan erradicar o inhibir las biopelículas. Estos compuestos actúan en diferentes etapas de la formación de la biopelícula e incluyen la inhibición de la adhesión, la inhibición del QS, o promueven estrategias de dispersión y mecanismos de disrupción de la matriz. Algunos de estos compuestos se resumen en la Tabla 1.

En esta tesis, investigamos la actividad antimicrobiana y antibiopelícula de un extracto de fruta con actividad proteolítica, contra diferentes especies de bacterias Gram-positivas y Gram-negativas (Manuscrito 1).

Tabla 1. Dianas y moléculas para controlar las biopelículas.

Etapa o estructura afectada	Moléculas	Mecanismo de acción
Adhesión	Extracto de *Cocculus trilobus*	Escinde las proteínas de superficie de las bacterias Gram-positivas
	Polifenoles de arándano	Interrumpe los factores de virulencia implicados en la adhesión, la acidogenicidad y la síntesis de glucanos
	Extracto de *Ginkgo biloba*	Reprime los genes curli y reduce la producción de fimbrias
	Ploretina	Reduce la producción de fimbrias, curli y genes que codifican toxinas
Quorum sensing	Furanona	Una molécula antagonista que suprime el QS y la motilidad bacteriana
	Quercetina	Reduce la expresión de LAsI, LasR, Rh1I y Rh1R que participan en QS
	Curcumina	Inhibe la AHL sintasa necesaria para QS
Matriz	Dispersina B	Degradación de PNAG por hidrólisis de los enlaces β-1,6 glicosídicos
	Dispersina B y α-amilasa	Degradación del eDNA
	Proteinasa K, quimotripsina, serratiopeptidasa	Proteasas implicadas en la degradación de la matriz
	carboxipeptidasa A, alginato liasa	
	Tripsina, papaina, actinidina	Proteasas implicadas en la degradación de la matriz y las fimbrias
	Fagos	Sus enzimas degradadoras de polisacáridos pueden destruir la matriz
	Miricetina	Inhibe la expresión de los genes implicados en la síntesis de EPS
Dispersión	BdcA	Se une a los c-di-GMP libres inhibiendo su papel en el mantenimiento de las biopelículas
	Ácido Cis-2-decenoico	Actividad de dispersión contra las biopelículas de diferentes patógenos
	AMP 1018	Suprime la respuesta regulada por la alarmona ((p)ppGpp)

EPS: Sustancias poliméricas extracelulares; QS: Quorum sensing; PNAG: Poli-β-(1,6)-N-acetilglucosamina; eDNA: Ácido desoxirribonucleico extracelular; c-di-GMP: diguanilato cíclico celular.

4. Determinantes de virulencia

El término virulencia se define como la capacidad de un organismo para infectar a un huésped susceptible y causar una enfermedad. Algunos determinantes de virulencia están implicados en la adherencia, la evasión del sistema inmunitario del huésped, la captación de hierro y la secreción de toxinas. Esta tesis analiza algunos de los principales determinantes de virulencia de *K. pneumoniae* y *E. coli* extraintestinales y su relación con la formación de biopelículas (Artículos 3 y 4). Aunque ambas especies comparten varios factores de virulencia, otros son únicos.

Dentro de los factores de virulencia encontrados tanto en las cepas de *K. pneumoniae* como en las de *E. coli* tenemos:

- Fimbrias de tipo 1: Son orgánulos filamentosos que se expresan en la superficie de la célula bacteriana y participan en la adhesión. Están formadas principalmente por subunidades FimA, con la subunidad FimH en la zona apical. Se expresan en el 90% de los aislados clínicos y ambientales de *K. pneumoniae* y en casi todas las *Enterobacterales*.

- Sideróforos: Son pequeñas moléculas que quelan el hierro, eleento esencial para los procesos metabólicos, el crecimiento y la replicación de las bacterias. Los sideróforos roban el hierro de las proteínas quelantes del huésped o lo capturan del medio ambiente. Tanto la enterobactina, la aerobactina, la yersiniabactina, como la salmochelina se han detectado en *K. pneumoniae* y *E.coli*.

- Polisacárido capsular (antígeno K): La cápsula bacteriana es una estructura extracelular que recubre la capa externa de la pared celular. Su función principal es proteger a las bacterias de diferentes tensiones físicas, químicas y biológicas. Participa en la formación de biopelículas y ayuda a las bacterias Gram-negativas a eludir la fagocitosis, la resistencia al suero y la opsonización.

- Lipopolisacárido (LPS) (antígeno O): En las bacterias Gram-negativas, el LPS es una de las moléculas estructurales más importantes de la membrana externa. El LPS consta de tres dominios: el lípido hidrofóbico A, el polisacárido del núcleo y los oligosacáridos de cadena larga (antígeno O-específico). El antígeno O es el componente más externo del LPS y consiste en un polímero de unidades de oligosacáridos repetidos. Es una de las estructuras celulares más variables debido a la variación de los azúcares presentes, lo que da lugar a una gama de antígenos O serológicamente distintos.

- Toxinas: Las toxinas bacterianas son factores de virulencia producidos por una variedad de patógenos que ayudan a que el patógeno se extienda a tejidos más profundos tras alterar la integridad celular, acceder a los nutrientes dentro de las células del huésped o escapar de la respuesta inmunitaria. Una importante toxina es el **factor citotóxico necrosante 1** (CNF1), que conduce a cambios celulares en el huésped tales como reordenamientos del citoesqueleto, detención del ciclo celular e interrupción de las vías de señalización. Otra de las toxinas destacadas es la **α-hemolisina** (HlyA), una toxina que forma poros en la membrana celular de los eritrocitos del huésped. La lisis osmótica se desencadena por la pérdida de iones K^+ intracelulares y la consiguiente entrada de cationes y agua. Finalmente, debemos nombrar la **colibactina**, un metabolito secundario que induce roturas de la doble cadena del ADN, aberraciones cromosómicas y la detención del ciclo celular en la fase G2/M. La isla genómica *pks* que la codifica se observa con frecuencia en las cepas de *E. coli* del filogrupo B2 y se ha encontrado en aislados de la microbiota intestinal, así como en aislados causantes de septicemia, meningitis neonatal e infecciones urinarias.

Un factor de virulencia específico de *K. pneumoniae* es la fimbria de tipo 3. Se expresan en la mayoría de los aislados clínicos de *K. pneumoniae* y median en la adhesión y la formación de biopelículas. La mayor parte de la

estructura consiste en subunidades MrkA, con la unidad de adhesión MrkD en la parte apical.

Existen otros factores de virulencia que son específicos de *E. coli*. Entre ellos tenemos:

- Fimbrias curli: Están compuestas por numerosas subunidades CsgA, que después de ser transportadas a través de la membrana externa, se polimerizan con la ayuda de la subunidad CsgB para formar la fibra funcional expuesta a la superficie. Las fimbrias curli son producidas por *E. coli* y *S. enterica* serovar Typhimurium en condiciones de limitación de nutrientes, oxígeno reducido y temperatura < 30 °C.

- Fimbrias P: Las fimbrias P median la adhesión de *E. coli* a las células urinarias epiteliales y a los eritrocitos. Por lo tanto, las fimbrias P están clásicamente asociadas a pielonefritis y a infecciones del torrente sanguíneo.

- Fimbrias S: Aunque las fimbrias de tipo 1 o P son predominantes en las *E. coli* patógenas extraintestinales (ExPEC), las fimbrias S también se encuentran en estas bacterias, pero con menor frecuencia. Las fimbrias S, o las fimbrias específicas del ácido siálico, están codificadas por el operón *sfa* que consta de nueve genes. Se han encontrado con mayor frecuencia en aislados de *E. coli* asociados a infecciones urinarias, meningitis y sepsis.

- Poli-β-(1,6)-N-acetilglucosamina (PNAG): El exopolisacárido PNAG es una adhesina que desempeña un papel crucial en la formación de biopelículas, la patogenicidad y la virulencia. Es uno de los polisacáridos más importantes en las biopelículas de *E. coli* e interviene en la adhesión célula-célula y en la adhesión a superficies.

- Flagelos (antígeno H): El flagelo es un orgánulo locomotor que permite a las bacterias desplazarse de entornos pobres en nutrientes a entornos ricos en nutrientes. Los flagelos permiten el movimiento en línea recta mediante la rotación de los filamentos en sentido contrario

a las agujas del reloj (modo de natación), o, por el contrario, en el modo de viraje, las bacterias rotan sus cuerpos en el sentido de las agujas del reloj.

5. Terapia antimicrobiana y resistencia a los antibióticos

El descubrimiento de los antibióticos fue uno de los acontecimientos más importantes de la historia de la humanidad. Durante décadas, los antibióticos han salvado muchas vidas y han ayudado a controlar las enfermedades infecciosas, una causa importante de morbilidad y mortalidad en todo el mundo. Los antibióticos tienen dianas específicas como: (1) la pared celular bacteriana, (2) la membrana celular, (3) la síntesis de proteínas, (4) la síntesis de ADN y ARN y, (5) el metabolismo del ácido fólico. Sin embargo, las bacterias utilizan diversas estrategias para evadir el ataque de los antibióticos. Algunas de estas estrategias son: (1) bombas de eflujo, que transportan los antibióticos desde el interior al exterior de la célula bacteriana; (2) modificaciones de la diana, que impiden la unión eficiente del antibiótico, pero permiten que la diana realice su función normal; (3) enzimas inactivadoras, que pueden inhibir o modificar varios antibióticos; (4) inmunidad y derivación, donde los antibióticos o sus dianas se unen a proteínas que impiden la unión entre el antibiótico y su diana.

Además, las bacterias pueden adquirir ADN externo que codifique para genes de resistencia y transferirlo a otras bacterias mediante transferencia horizontal. Este proceso puede ocurrir a través de varios mecanismos: transformación (mediante ADN libre), transducción (mediante bacteriófagos) o conjugación (mediante plásmidos).

Algunos de los agentes antimicrobianos más utilizados para tratar las infecciones causadas por *E. coli* y *K. pneumoniae*, se explican brevemente a continuación:

- Aminoglucósidos: Pertenecen al grupo de antibióticos que inhiben la síntesis de proteínas al unirse de forma irreversible y con gran afinidad al sitio A de la subunidad ribosomal pequeña 30S. Son

activos contra la mayoría de las bacterias Gram-negativas aerobias, pero carecen de actividad significativa contra *Burkholderia cepacia, Stenotrophomonas maltophilia, Pasturella multocida* y organismos anaerobios. Las bacterias Gram-positivas son relativamente resistentes a los aminoglucósidos, por lo que su uso en estos microorganismos es limitado.

- Quinolonas: Son los agentes antimicrobianos sintéticos más eficaces con actividad de amplio espectro que inhiben las topoisomerasas bacterianas. Las quinolonas actúan sobre la ADN girasa y la topoisomerasa IV bacterianas, que son cruciales para la replicación, la transcripción, la recombinación y la remodelación del ADN condensado. En consecuencia, las quinolonas se asocian a la inhibición de la síntesis de ADN y ARN.

- Trimetoprim-sulfametoxazol: Es un agente antimicrobiano sintético de amplio espectro. Ambos fármacos afectan a la síntesis del ácido fólico bacteriano al inhibir dos de las enzimas clave de esta vía. Así, el sulfametoxazol inhibe la dihidropteroato sintetasa, que cataliza la formación de dihidrofolato a partir del ácido paraaminobenzoico; y el trimetoprim tiene un efecto inhibidor sobre la dihidrofolato reductasa, que cataliza la formación de tetrahidrofolato a partir del dihidrofolato.

- Cloranfenicol: Es un antibiótico bacteriostático de amplio espectro producido naturalmente por *Streptomyces venezuelae*. Inhibe la biosíntesis de proteínas al interactuar con la subunidad 50S del ribosoma bacteriano y bloquear la unión del aminoacil-ARNt en el sitio A.

- Fosfomicina: Es un antibiótico bactericida de amplio espectro producido por *Streptomyces* spp. que afecta a la biosíntesis del peptidoglicano. Una vez en el interior de la célula, la fosfomicina inhibe la enzima UDP-N-acetilglucosamina enolpiruvil transferasa (MurA), responsable de catalizar la formación de ácido N-

acetilmurámico (precursor del peptidoglicano) mediante la unión de N-acetilglucosamina y fosfoenolpiruvato.

- Colistina: También conocida como polimixina E, pertenece a la familia de antibióticos de las polimixinas, un grupo de oligopéptidos antimicrobianos cíclicos con propiedades hidrofílicas y lipofílicas, que comprende cinco compuestos (A, B, C, D y E). Es eficaz contra las bacterias Gram-negativas pero ineficaz contra las bacterias Gram-positivas, las bacterias anaerobias y los micoplasmas. Se considera el agente antimicrobiano de última elección para las infecciones causadas por bacterias MDR. La colistina se une a los grupos fosfato del lípido A presente en el LPS de las bacterias Gram-negativas mediante interacciones electrostáticas, destruyendo la integridad de las membranas externas e internas de las bacterias.

- β-Lactámicos: El anillo β-lactámico interactúa con la serina nucleófila de las proteínas de unión a la penicilina (PBP), lo que conduce a la acilación irreversible de las PBP, que impide la formación de enlaces transpeptídicos de peptidoglicano. Existen cinco clases principales de β-lactámicos antimicrobianos: penicilinas, cefamicinas, cefalosporinas, carbapenemicos y monobactamicos.

- Inhibidores de β-lactamasas: El ácido clavulánico fue el primer inhibidor identificado contra la mayoría de las enzimas de clase A que se encuentran en *Enterobacterales*. Tras el descubrimiento del ácido clavulánico, se aprobaron con éxito el sulbactam y el tazobactam en combinación con varios β-lactámicos. Sin embargo, su limitado espectro de actividad ha impulsado el desarrollo de nuevos inhibidores. Entre ellos, el avibactam y el uso de meropenem en combinación con el boronato cíclico vaborbactam. En los últimos años se han descubierto dos moléculas de diazabiciclooctano: el relebactam y el nacubactam.

6. Justificación de la tesis, hipótesis y objetivos

Las biopelículas son un gran problema sanitario mundial debido a su inherente tolerancia a los tratamientos antimicrobianos. Aunque algunas de ellas son monomicrobianas, muchas otras son comunidades polimicrobianas, en las que se incluyen ciertas infecciones humanas. Los mecanismos subyacentes a la formación de biopelículas no se conocen en profundidad, y muchos genes de virulencia o incluso genes de resistencia a los antimicrobianos podrían estar implicados en este proceso. La identificación de dichos genes como posibles objetivos de terapias antibiopelícula, así como el descubrimiento de nuevas moléculas para inhibir o erradicar las biopelículas es actualmente un objetivo prioritario en investigación.

Por ello, esta tesis se centra en el estudio de la formación de biopelículas desde diferentes ángulos. En primer lugar, hemos analizado las interacciones entre especies en las infecciones del tracto urinario, concretamente en la biopelícula formada por *K. pneumoniae* y *E. faecalis*. En segundo lugar, hemos buscado nuevos genes implicados en la formación de biopelículas en *E. coli* mediante el uso de mutagénesis aleatoria por inserción de un transposón. También hemos investigado una nueva molécula de origen natural que podría considerarse en el futuro como agente para tratar las biopelículas formadas no sólo por *E. coli*, sino también por otras bacterias Gram-negativas y Gram-positivas. Por último, hemos analizado si el origen de las cepas, los perfiles de resistencia a los antimicrobianos o la presencia o ausencia de diferentes determinantes de virulencia en una colección de cepas de *E. coli* y *K. pneumoniae* podrían estar relacionados con su capacidad para formar biopelículas. Por tanto, en esta tesis se han planteado cuatro hipótesis y objetivos:

Hipótesis 1

Las interacciones antagónicas modulan la biopelícula polimicrobiana formada por *K. pneumoniae* y *E. faecalis*.

Objetivo 1

Analizar las interacciones interespecies que ocurren dentro de la biopelícula formada por *K. pneumoniae* y *E. faecalis* (Artículo 1).

Hipótesis 2

El método de mutagénesis de inserción aleatoria podría facilitar el descubrimiento de nuevos genes implicados en la formación de biopelículas de *E. coli*, que podrían luego considerarse como nuevas dianas terapéuticas.

Objetivo 2

Identificar nuevos genes implicados en la formación de biopelículas en *E. coli* mediante el análisis transcriptómico y proteómico de mutantes isogénicos generados por transposición (Artículo 2).

Hipótesis 3

Nuevas moléculas de origen natural podrían tener un efecto bactericida y antibiopelícula, ayudando a combatir tanto la resistencia a los antibióticos como la formación de biopelículas.

Objetivo 3

Evaluar la actividad antibacteriana y antibiopelícula de un extracto de fruta que contiene enzimas proteolíticas contra diversas especies de bacterias Gram-positivas y Gram-negativas (Manuscrito 1).

Hipótesis 4

El origen de las cepas bacterianas, sus perfiles de resistencia antimicrobiana y la presencia o ausencia de diferentes genes de virulencia podrían estar relacionados con la formación de biopelículas.

Objetivo 4

Caracterizar una colección de cepas clínicas de *E. coli* y *K. pneumoniae* en cuanto a su resistencia a los antimicrobianos y determinantes de virulencia

para dilucidar una relación entre estas características y la capacidad de formar biopelículas (Artículo 3 y Artículo 4).

7. Resultados y discusión

En el primer objetivo, nos hemos centrado en evaluar las interacciones interespecíficas de las biopelículas polimicrobianas de *K. pneumoniae* y *E. faecalis*, ya que esta interacción ha sido poco estudiada hasta ahora y son dos patógenos frecuentemente encontrados como agentes causales de infecciones urinarias. En este contexto, encontramos una dominancia de *E. faecalis* sobre *K. pneumoniae*, apoyada por el índice competitivo (IC) y la cuantificación de células viables, indicando una relación antagónica entre las dos especies y un efecto inhibidor de *E. faecalis* sobre *K. pneumoniae*. Dicho antagonismo se observó al utilizar caldo tripticasa de soja u orina humana enriquecidos con glucosa. Sin embargo, interacciones neutras fueron observadas cuando el medio en el que se desarrolló la biopelícula no contenía glucosa, lo que sugiere que podría existir una diferencia en las interacciones entre ambas especies en pacientes diabéticos y no diabéticos, aunque esta hipótesis necesita de un estudio más profundo.

Se sabe que las bacterias producen diferentes compuestos que pueden interferir en el crecimiento de otras especies, como peróxido de hidrógeno, ácidos orgánicos o bacteriocinas. En este caso, descartamos la producción de bacteriocinas porque observamos efectos antibacterianos y antibiopelícula sólo con sobrenadantes liofilizados libres de células cuyo pH no fue ajustado. Por este motivo, nos centramos en el estudio de la producción de ácidos orgánicos como causa de la inhibición de *K. pneumoniae*. En nuestro caso, cuando se midieron los niveles de ácido láctico en los sobrenadantes, la concentración fue suficiente para inhibir el crecimiento y desarrollo de la biopelícula de *K. pneumoniae*, siendo, por tanto, el agente causal de la inhibición.

Para confirmar nuestra hipótesis, decidimos utilizar cepas mutantes de *E. faecalis* defectuosas en la producción de ácido láctico. La reducción del número de colonias de *K. pneumoniae* no fue significativa cuando se utilizaron

las cepas mutantes de *E. faecalis* V583 *Δldh-1* o *Δldh-1/Δldh-2* en la formación de la biopelícula polimicrobiana. Esto confirma que la producción de ácido láctico podría dar a *E. faecalis* una ventaja sobre *K. pneumoniae* u otras especies, ya que algunas cepas de *E. faecalis* pueden adaptarse a diferentes rangos de pH, creciendo así en condiciones altamente ácidas.

Todos estos hallazgos nos llevan a seguir investigando las bacterias lácticas como posibles agentes de control biológico en la formación de biopelículas, lo que nos lleva a nuevos enfoques en las medidas de prevención.

Siguiendo con la búsqueda de nuevas estrategias para controlar las biopelículas, en el artículo 2 utilizamos la técnica de inserción de transposones al azar para encontrar nuevos genes implicados en su formación en *E. coli*, que podrían llegar a ser diana de tratamiento antibiopelícula. Creamos una biblioteca de mutantes al azar de las que se analizaron 20 de ellas, las cuales mostraban una capacidad reducida en la formación de biopelículas. La caracterización fenotípica de estas cepas se realizó mediante los ensayos de swimming, rojo congo y hemaglutinación. A partir de los resultados obtenidos, se seleccionaron las cepas mutantes Tn263 y Tn463 para la secuenciación del genoma completo.

En la cepa Tn463, el transposón se insertó en los genes *csgA* y *cysB*. Dado que el gen *csgA* se ha asociado previamente con la formación de biopelículas, este mutante se descartó para los siguientes análisis. El gen *csgA* codifica las subunidades CsgA, componente principal de las fibras amiloides de curli, esenciales en la fase de adhesión. En la cepa mutante Tn263, el transposón se insertó en el gen *purL*, que codifica la 5'-fosforibosil-formil-glicinamida amidotransferasa, implicada en la vía biosintética de novo de las purinas. Los análisis de las cepas Tn263, la mutante *ΔpurL::cat* y la cepa complementada *ΔpurL/purL+*, mostraron que *ΔpurL::cat*, al igual que la cepa mutante Tn263, también había perdido la capacidad de formar biopelículas y dio lugar a colonias de color rosa claro asociadas a fenotipos de curli defectuosos en agar rojo congo. La cepa complementada *ΔpurL/purL+* mostró valores de absorbancia similares a los de la cepa salvaje y restauró su

capacidad de formar fimbrias curli y biopelículas. Además, las cepas Tn263 y *ΔpurL::cat* recuperaron su capacidad de formar biopelículas cuando se añadió inosina monofosfato (IMP) al medio.

Estos resultados demuestran el papel crucial del gen *purL* y de la vía de biosíntesis de purinas en la producción de fimbrias curli y, por tanto, en la producción de biopelículas.

Los estudios proteómicos adicionales en las cepas wild-type (wt) y Tn263 revelaron diferencias en 13 proteínas. Las diferencias más significativas se observaron en las proteínas DnaK, GroEL, Pta, PtsI y OmpA, la mayoría de ellas asociadas previamente a la formación de biopelículas. Nuestro análisis transcriptómico reveló que tres genes estaban infraexpresados (*dnaK, groL* y *adhE*), seis genes estaban sobreexpresados (*lptD, cysJ, pta, ilvC, elbB* y *ptsI*) en el mutante Tn263 en comparación con la cepa wt, y cuatro genes mostraron niveles de expresión similares entre ambas cepas (*tsf, ftsZ, ompA* y *purL*). Los genes con diferentes niveles de expresión también se analizaron en cepas clínicas formadoras de biopelículas y no formadoras de biopelículas. Aunque descubrimos que los genes *adhE* y *ptsI* estaban sobreexpresados en las cepas clínicas no formadoras de biopelículas, no hubo concordancia entre los resultados transcriptómicos y proteómicos de la cepa mutante Tn263 y la comparación con las cepas no formadoras de biopelículas. Por lo tanto, concluimos que las diferencias en la expresión génica en la cepa mutante Tn263 pueden estar relacionadas, exclusivamente, con la mutación del gen *purL* y no con el fenotipo de no formación de biopelícula *per se*.

En este sentido, nuestros resultados proporcionan una fuerte evidencia de que el gen *purL* está implicado en la formación de biopelículas dependiente de curli. Por tanto, los genes involucrados en las vías biosintéticas de nucleótidos pueden ser excelentes candidatos para potenciales compuestos antibiopelícula.

Después del hallazgo de una nueva y potencial diana para el tratamiento de las biopelículas formadas por *E. coli*, quisimos analizar un

extracto de fruta con actividad antibacteriana y antibiopelícula, en vista también de la preocupación mundial por la resistencia a los antimicrobianos. En el manuscrito 1, resumimos los resultados obtenidos al estudiar un extracto de fruta con actividad proteolítica contra diferentes especies bacterianas, incluyendo cepas Gram-positivas y Gram-negativas. Todas las cepas eran formadoras de biopelículas y estaban implicadas en diferentes tipos de infección. Descubrimos que este extracto de fruta mostraba un efecto antibacteriano y era capaz de destruir biopelículas maduras. Además, demostramos su efecto bactericida a 2x CMI y 4x CMI tras 24 horas de incubación. Sin embargo, el valor IC50 obtenido mediante el ensayo de XTT en células Jurkat para determinar la citotoxicidad del extracto es impreciso debido a la viscosidad y turbidez del mismo.

Nuestro extracto mostró una gran actividad contra algunas de las especies implicadas en la enfermedad periodontal, entre ellos *E. faecalis*, S. mutans, *E. coli* y *K. pneumoniae*. Por lo tanto, la molécula activa podría probarse para ser usada en pastas de dientes o colutorios, para prevenir y combatir la placa dental y otras enfermedades bucodentales. De igual manera, el extracto mostró actividad frente a Salmonella sp. y Shigella sp., dos especies relacionadas con enfermedades intestinales incluyendo biopelículas en la vesícula biliar.

Aunque los valores de CMI obtenidos (que oscilaron entre 35 mg/mL y 50 mg/mL) y MBEC (que oscilaron entre 150 mg/mL y 400 mg/mL) son alentadores, éstos podrían mejorarse si pudiéramos probar la molécula activa aislada del extracto. Una vez conseguido esto, el extracto de fruta con enzimas proteolíticas podría ser un nuevo y prometedor agente antimicrobiano y antibiopelícula.

Sin embargo, dada la dificultad que conlleva el desarrollo de un nuevo antimicrobiano, quisimos explorar también otras estrategias que vincularan el estudio de genes involucrados en virulencia y resistencia con la formación de biopelículas, lo que también podría ser eficaz para encontrar otras dianas para combatirlas. El estudio de las biopelículas formadas por bacterias del grupo ESKAPE y de la lista de patógenos prioritarios de la Organización

Mundial de la Salud, está despertando un interés creciente. *K. pneumoniae* y *E. coli* son dos bacterias Gram-negativas que pertenecen a esta lista y están implicadas en numerosas infecciones. Por tanto, la caracterización de las cepas de estas especies en términos de virulencia y resistencia puede ayudar a identificar nuevos factores implicados en la formación de biopelículas, facilitando la búsqueda de nuevas estrategias para inhibirlas o erradicarlas.

En el artículo 3, determinamos la presencia de una serie de genes de virulencia y resistencia entre 127 cepas clínicas de *K. pneumoniae* aisladas de orina, tracto respiratorio y sangre. También se determinó la resistencia al suero, la prueba de hipermucoviscosidad y la formación de biopelículas.

Nuestros resultados mostraron que fimbrias y enterobactina están presentes en prácticamente todas las cepas clínicas de *K. pneumoniae*. Además, se observó un porcentaje creciente del sideróforo yersiniabactina en comparación con otros estudios. También encontramos el gen *rmpA* (7,1%) y el fenotipo hipermucoviscoso (13,4%) en una proporción significativa entre las cepas analizadas en nuestro estudio.

Como se ha observado en estudios anteriores, encontramos una prevalencia considerable de genes codificadores de yersiniabactina entre los aislados del tracto respiratorio, lo que confirma el papel crucial de la yersiniabactina en las infecciones pulmonares. Además, encontramos una asociación estadísticamente significativa entre el gen asociado a la cápsula *uge* (UDP galacturonato 4-epimerasa) y el origen de los aislados, con la mayoría de los aislados procedentes de orina portando este gen.

Es importante destacar el aumento global de las cepas hipervirulentas de *K. pneumoniae* (hvKp) y su impacto en la salud. En el presente trabajo, clasificamos 24 cepas (18,9%) como hvKp. De ellas, 13 cepas eran sensibles a todos los antimicrobianos probados, nueve eran productoras de β-lactamasas de espectro extendido (BLEE), una era resistente a la fosfomicina y una era multirresistente (MDR). Otra preocupación creciente en la investigación sanitaria es el alarmante aumento de bacterias Gram-negativas portadoras de colibactina. Aunque en nuestro estudio sólo encontramos dos cepas positivas

a la colibactina, esta toxina se está investigando en varias especies por su asociación con el daño producido en el ADN y la alteración del ciclo celular del huésped.

En cuanto a la formación de biopelículas, varios estudios han encontrado un porcentaje similar o incluso mayor de cepas formadoras de biopelículas que las observadas por nosotros (80,3%), lo que sugiere que las cepas clínicas de *K. pneumoniae* tienen una alta capacidad de formar biopelículas. Aunque algunos estudios no encontraron ninguna correlación entre la formación de biopelículas y el origen de los aislados, nosotros encontramos que las cepas de orina formaban más biopelícula que los aislados del torrente sanguíneo o del tracto respiratorio. También observamos que las cepas hipermucoviscosas tenían una menor capacidad para formar biopelículas. Este hecho puede explicarse por la capacidad de la hipercápsula de ocultar las fimbrias de tipo 3 y 1, impidiendo así la adhesión.

En cuanto a la resistencia a los antimicrobianos, encontramos un 40% de cepas con fenotipo multirresistente, siendo casi el 50% aisladas de orina. Asimismo, el 43,3% de las cepas eran productoras de BLEE y el 10,2% eran productoras de carbapenemasas. Dentro de las cepas productoras de BLEE, las cepas de orina produjeron más este tipo de enzimas que las cepas respiratorias o sanguíneas.

Luego de los resultados observados con la colección de cepas de *K. pneumoniae*, caracterizamos 376 cepas extraintestinales de *E. coli* (ExPEC), cuyos resultados se encuentran en el artículo 4. Los perfiles de resistencia a los antimicrobianos en nuestra colección mostraron los mayores porcentajes de resistencia a ciprofloxacina (48,7%), trimetoprim-sulfametoxazol (47,9%) y ampicilina (38%), encontrando igualmente un porcentaje significativo de cepas con genes que codifican BLEE, siendo $bla_{CTX\text{-}M\text{-}15}$ la más común.

Nuestros resultados mostraron que el 38% de los aislados se clasificaron como MDR. Sin embargo, encontramos una correlación directa entre la susceptibilidad antimicrobiana y la capacidad de formar biopelículas, pero esta correlación no fue estadísticamente significativa. De manera

interesante, las cepas no formadoras de biopelículas tenían un mayor porcentaje de resistencia a la ciprofloxacina (74,6%) en comparación con las cepas formadoras de biopelículas, lo que ya ha sido previamente observado en otros estudios.

En cuanto a la clasificación por grupos filogenéticos, los aislados pertenecientes a los grupos filogenéticos B2 y D mostraron una mayor capacidad de formación de biopelículas que los grupos considerados comensales. Sin embargo, a diferencia de lo que ocurrió con las cepas de *K. pneumoniae* analizadas previamente, no observamos ninguna relación entre el origen de las cepas y la formación de biopelículas.

El 84,3% de las cepas eran capaces de formar biopelículas y esta característica esta significativamente relacionada con la presencia de varios sideróforos, adhesinas o toxinas. El gen *iroN*, que codifica el receptor de la salmochelina, el gen de la fimbria de tipo 1 (*fimH*), los genes *sfa* y *papA* que codifican adhesinas, y los genes *hlyA* y *cnf1* que codifican para hemolisina y el factor citotóxico necrosante 1 respectivamente, estaban estadísticamente correlacionados con la formación de biopelículas.

Uno de los hallazgos más notables de nuestro estudio fue la relación entre los genes que codifican para colibactina y la formación de biopelículas. Así, un gran porcentaje de cepas de *E. coli* fueron positivas para colibactina (19,9%), la mayoría de ellas eran formadoras de biopelículas, pertenecientes al grupo filogenético B2, con una tasa de resistencia a los antimicrobianos bastante baja, una gran diversidad clonal y una alta relación con la mayoría de los factores de virulencia estudiados. Esta toxina se ha relacionado con el cáncer colorrectal en humanos, y algunos estudios también han demostrado su papel crucial en la patogénesis de las infecciones de tracto urinario al inducir daños en el ADN de las células uroteliales, así como en casos de meningitis.

El papel de la colibactina en las biopelículas y su importancia en diversas enfermedades infecciosas requiere más investigación. Las biopelículas formadas por *E. coli* y otros patógenos prioritarios también

requieren más estudios, ya que en este proceso pueden intervenir factores ambientales u otros factores genéticos aún no identificados.

Adicionalmente, la vigilancia epidemiológica es una necesidad no sólo para detectar la resistencia a los antibióticos, sino también los factores de virulencia, con el fin de prevenir la propagación de cepas con patotipos hipervirulentos que suponen una amenaza real para las poblaciones susceptibles, especialmente cuando estas cepas son portadoras de genes de resistencia a los antimicrobianos.

8. Conclusiones

1. El índice competitivo (IC) y la cuantificación de células viables indican una interacción competitiva entre *E. faecalis* y *K. pneumoniae* en biopelículas polimicrobianas cuando se co-cultivan en un medio enriquecido con glucosa.

2. Aunque *K. pneumoniae* tiene una mayor capacidad de adhesión que *E. faecalis*, esta propiedad no es decisiva para el desarrollo posterior de la biopelícula polimicrobiana formada por ambas especies.

3. El efecto inhibidor de *E. faecalis* sobre *K. pneumoniae* no se debió a la producción de bacteriocinas, sino al ácido láctico producido por *E. faecalis*, el cual inhibió el crecimiento de *K. pneumoniae* en condiciones de exceso de glucosa y oxígeno limitado.

4. El extracto de fruta mostró actividad antibacteriana y fue capaz de disgregar las biopelículas maduras formadas por diferentes especies de bacterias Gram-positivas y Gram-negativas, por lo que, de poder aislarse la molécula activa de éste extracto, podríamos estar ante un buen candidato a agente antibiopelícula.

5. El gen *purL* es esencial para la formación de biopelículas mediada por fimbrias curli en *E. coli*, y podría considerarse una potencial diana para tratamientos antibiopelículas.

6. Las cepas mutantes Tn263 y *ΔpurL::cat* perdieron la capacidad de formar biopelículas mediadas por fimbrias curli en el caldo M63. Sin embargo, las cepas recuperaron esta capacidad cuando el medio se enriqueció con inosina.

7. La inserción del transposón se localizó en el dominio N-terminal del gen *purL*, afectando la biosíntesis de las purinas y la producción de biopelículas dependientes de fimbrias curli. Igualmente, la expresión de 13 proteínas, incluyendo AdhE, CysJ, DnaK, ElbB, FtsZ, GroL, IlvC, LpD, MgtA, OmpA, Pta, PtsI y Tsf se vio afectada.

8. En la colección bajo estudio, las cepas de *K. pneumoniae* de origen urinario fueron más resistentes a los antimicrobianos, presentaron más BLEE y tenían mayor capacidad de formar biopelículas "in vitro" que las cepas respiratorias o sanguíneas.

9. En *E. coli*, el gen *uge* se asoció significativamente con las cepas de origen urinario, mientras que la yersiniabactina se asoció significativamente con las cepas aisladas del tracto respiratorio, lo que indica el papel determinante que tienen estos genes con el nicho en el que se encuentra la bacteria que lo porta.

10. Se observó una relación inversa entre la resistencia a los antimicrobianos y la virulencia en las cepas de *K. pneumoniae* y *E. coli* analizadas. Así, las cepas más resistentes presentaron una baja prevalencia de genes de virulencia y viceversa.

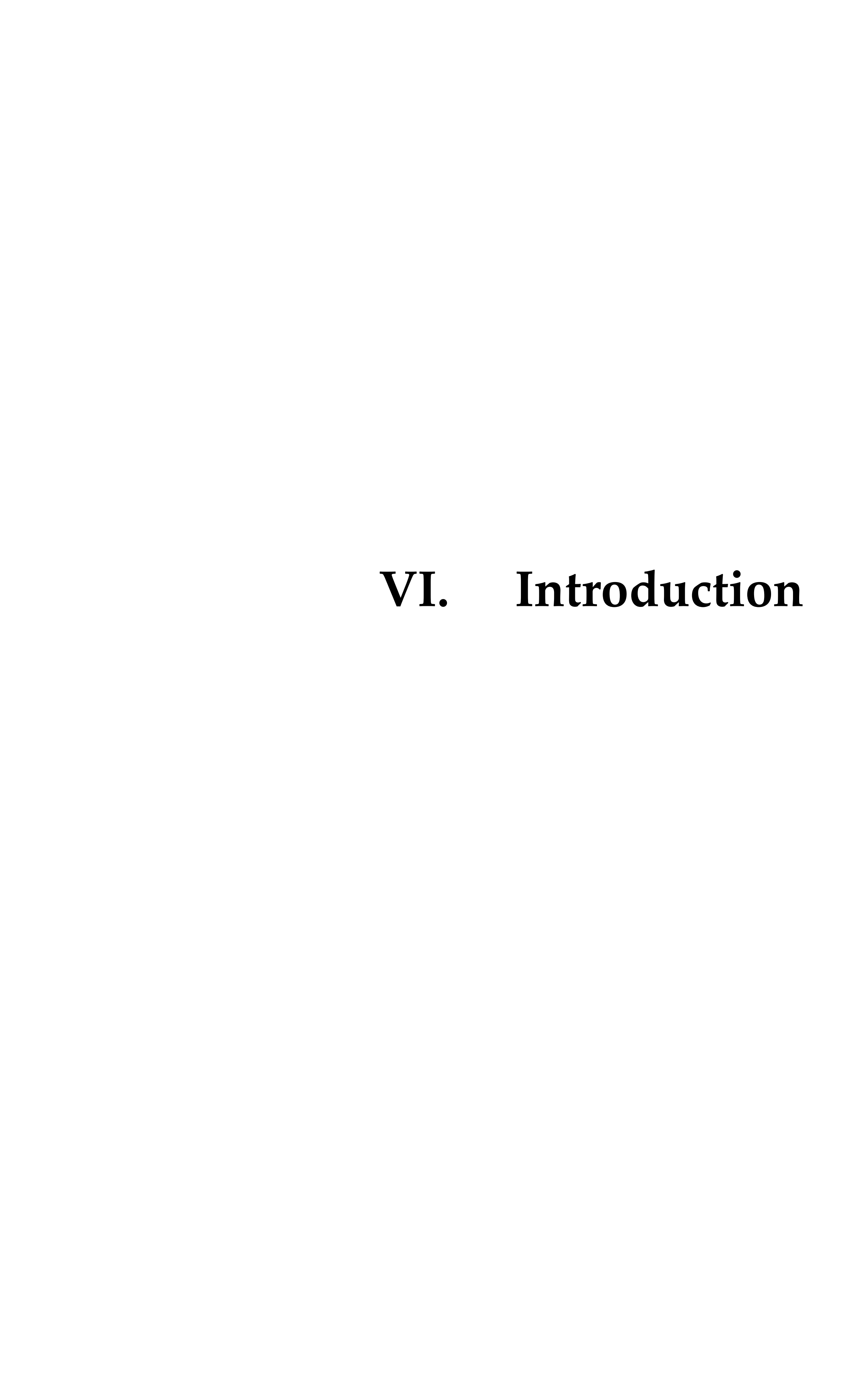
11. Las cepas de *E. coli* resistentes a la ciprofloxacina mostraron una menor capacidad de formación de biopelículas, lo que confirma los estudios previos realizados en nuestro laboratorio.

12. La presencia de genes que codifican para colibactina se correlacionó estadísticamente con la formación de biopelículas en las cepas de *E. coli*. Debido a que esta toxina está relacionada con el desarrollo de cáncer colorectal, un estudio más profundo de la relación entre la

presencia de esta toxina, la formación de biopelículas y a su vez la asociación con este tipo de cáncer sería de gran interés.

13. Las biopelículas formadas por *E. coli*, *K. pneumoniae* y otros patógenos prioritarios requieren estudios adicionales, ya que en el proceso de formación de biopelículas pueden intervenir factores genéticos aún no identificados.

14. La vigilancia epidemiológica debería ser obligatoria a nivel mundial, con el fin de detectar los clones circulantes que suponen una amenaza para las poblaciones susceptibles, especialmente cuando estos patotipos portan genes de virulencia y resistencia considerados de alto riesgo.



VI. Introduction

1. Characteristics and relevance of biofilms

1.1. Biofilms definition

Biofilms have been defined as aggregates of microbial cells embedded in a self-produced matrix of extracellular polymeric substances (EPS) (1). These cells can adhere to each other and also to a wide variety of biotic (meat, living tissue, oral cavity, intestine, genitourinary tract, skin) or abiotic (floors, walls, drains, appliances, medical devices, industrial plumbing systems, or natural aquatic systems) surfaces (2,3). Adhesion to a surface provides biofilms with environmental stability and protection from acids, desiccation, salinity, phagocytosis, and the action of various antimicrobial agents (4). All these factors make biofilm formation a successful bacterial survival strategy.

This thesis mainly focuses on biofilm formation of *Klebsiella pneumoniae* and *Escherichia coli*, two of the most significant Gram-negative pathogens associated with hospital- and community-acquired infections.

1.2. Stages of biofilm formation

Due to social and physical interactions between cells, the biofilm lifestyle differs considerably from planktonic bacterial cells. For example, the metabolic properties within biofilm communities are entirely different, leading to the evolution of a sophisticated architecture that provides the opportunity for metabolic cooperation in well-organised niches that do not exist in planktonic bacteria (5). This spatial organisation involves a complicated development process that takes place in different phases: adhesion, maturation, and dispersal.

1.2.1. Adhesion

In general, adhesion refers to the first interaction of bacteria with a surface (Figure 1). Living cells actively secrete chemicals to attach reversibly

in the early stages until adhesion becomes permanent (4). It has been proposed that the conversion from reversible to permanent attachment is a regulated process, perhaps allowing cells to analyse the local environment before moving to a sessile lifestyle (6). Physicochemical properties such as osmolarity, ionic strength, pH, and nutrient availability, play a significant role at this stage (7).

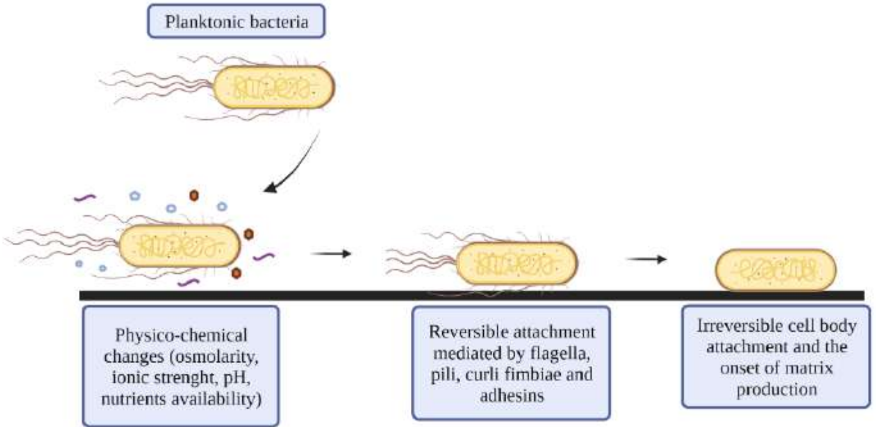


Figure 1. Bacterial adhesion to a surface in motile species. This figure was created with BioRender.com.

Adhesion depends on several factors, such as the surface and the medium in which it takes place. Additionally, each bacterial species shows particular characteristics at this stage (8). Thus, bacteria can produce fimbriae, curli, flagella, adhesins, and other structures that play an essential role in the initial phase of biofilm formation (3,9). In non-motile bacteria, the cells increase the expression of adhesins, which promotes cell-cell interactions and adhesion to the surface (10). However, once motile bacteria find a suitable surface, they lose their motility and produce the extracellular matrix.

In *E. coli* and *K. pneumoniae* species, type 1 fimbriae (filamentous organelles of bacterial adhesion) are responsible for irreversible attachment to surfaces in the process of biofilm formation and also enable attachment to various receptors on eukaryotic cells (11). It has been suggested that type 1 fimbrial lectins differ between and within bacterial species depending on

their sugar specificity. In this sense, *E. coli* and *K. pneumoniae* exhibit differences due to conformational changes in the FimH subunit, the mannose-binding moiety (12). Sokurenko *et al.* found that uropathogenic *E. coli* expressed type 1 fimbriae with specificity for monomannose and trimannose, whereas faecal isolates had specificity only for trimannose (13).

In the case of *E. coli*, motility appears to be important in the early stages of biofilm formation and depends on flagella, motile organelles that allow bacteria to respond to chemical signals (14). Flagella are necessary to swim in the medium in which the bacteria are immersed and spread on the surface (15,16). Conversely, in non-motile *E. coli* strains, the overproduction of amyloid fibres, known as curli fimbriae, allows adhesion and subsequent biofilm development without the intervention of flagella (16). Curli expression depends on factors that may influence bacterial attachment, such as temperatures below 30 °C, nutrient limitation, and the stationary growth phase (17). In both cases, biofilm cells become immobile.

Regarding *K. pneumoniae*, some of the major virulence factors contributing to biofilm formation are capsular polysaccharides, Kpc adhesin, and type 1 and type 3 fimbriae (18,19). Kpc fimbriae are closely related to the K1 serotype and hypermucoviscous phenotype and may be associated with biofilm formation (20). Type 3 fimbriae, found in most *K. pneumoniae* strains, are encoded by the *mrk* genes and provide the fimbrial binding ability that initiates biofilm formation (18,20).

Virulence factors related to biofilm formation are discussed in more detail in section 4.

1.2.2. Maturation

Once cells have irreversibly attached to the surface, the biofilm enters the maturation phase. At this stage, matrix production begins and allows the formation of more structured communities, determining the final architecture and spatial arrangement of the biofilm.

The matrix consists mainly of water (97%) and exopolysaccharides (mannose, galactose, and glucose are the most abundant, followed by N-acetyl-glucosamine, galacturonic acid, arabinose, fucose, rhamnose, and xylose) as well as proteins, nucleic acids, lipids, extracellular deoxyribonucleic acid (eDNA), and a large number of small molecules (Figure 2). This structure provides mechanical stability, transport of nutrients and waste, and protection from desiccation, antimicrobial agents, antibodies, and the effects of the host immune response, including complement action and phagocytosis (6,16,21–23).

Several factors are involved in the development of the matrix in *E. coli*. Under stress conditions, the production of colanic acid increases, contributing to a voluminous biofilm structure (17,23). Similarly, Ag43, an outer membrane protein belonging to the autotransporter family and encoded by the *flu* gene, promotes cell-cell adhesion and the development of a mature three-dimensional (3D) biofilm (16).

In this phase, quorum sensing (QS) plays a vital role. QS is an intercellular signalling process that enables communication between the bacteria present in the community or even between bacteria and the host (3). This communication is triggered by the production of small biochemical signalling molecules that induce the expression of genes encoding extracellular factors. The production of these factors is a cooperative trait that confers advantages to the community and promotes the regulation of other genes related to biofilm maturation and maintenance (24–26). The *lux*-type QS system is the most important factor among Gram-negative bacteria. It is mediated by the production of acyl-homoserine lactones (AHLs) and consists of two components, an autoinducer synthase (LuxI) and a transcriptional regulator (LuxR) (24). When AHL concentrations increase outside the cell, AHL diffuses across cell membranes, binding to and activating the LuxR receptor, promoting and regulating the differential expression of virulence factor genes (VFGs), such as lectin, elastase, proteases, toxins and others (24,27).

In addition to QS molecules, other signals trigger biofilm formation, including secondary metabolites, antibiotics, pigments, and siderophores (28). Thus, the mature biofilm becomes a complex, heterogeneous, three-dimensional (3D) structure in which the existing channels transport nutrients and oxygen to the entire bacterial community (29).

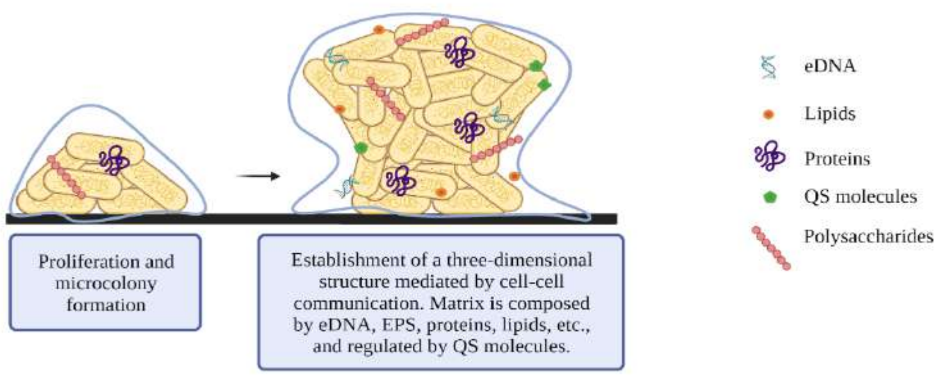


Figure 2. Biofilm maturation. This figure was created with BioRender.com.

1.2.3. Dispersal process

The dispersal process is the final stage of biofilm development. This stage promotes the spread of bacteria in the environment and subsequent colonisation of new surfaces. It can be influenced by environmental conditions, such as low nutrient availability, insufficient oxygen or pH levels, an increase in toxic product levels, endogenous enzymatic degradation or other stress conditions (4,6,8,30). Biofilm cell release is mediated by dispersion or detachment. The main difference between these two mechanisms is that **dispersion** is active, whereas **detachment** is passive.

Dispersion is an active process in which bacteria escape from the biofilm through enzymatic degradation, leaving eroded biofilms behind and allowing the bacteria to spread to new sites (Figure 3A) (31,32).

In contrast, in passive **detachment,** external factors such as fluid shear forces, abrasion, and external human disturbances act as inductors of this process (Figure 3B).

The second messenger, cellular bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), is one of the primary molecules involved in the dispersal process. Dispersion leads to a decrease in c-di-GMP, which contributes to the appearance of a heterogeneous population of dispersed cells, a phenotype distinct from sessile or planktonic cells. Dispersed cells typically have higher motility, virulence and adherence, and exhibit altered antimicrobial susceptibility patterns, leading to a phenotype between biofilm cells and planktonic cells, with different characteristics from both (31).

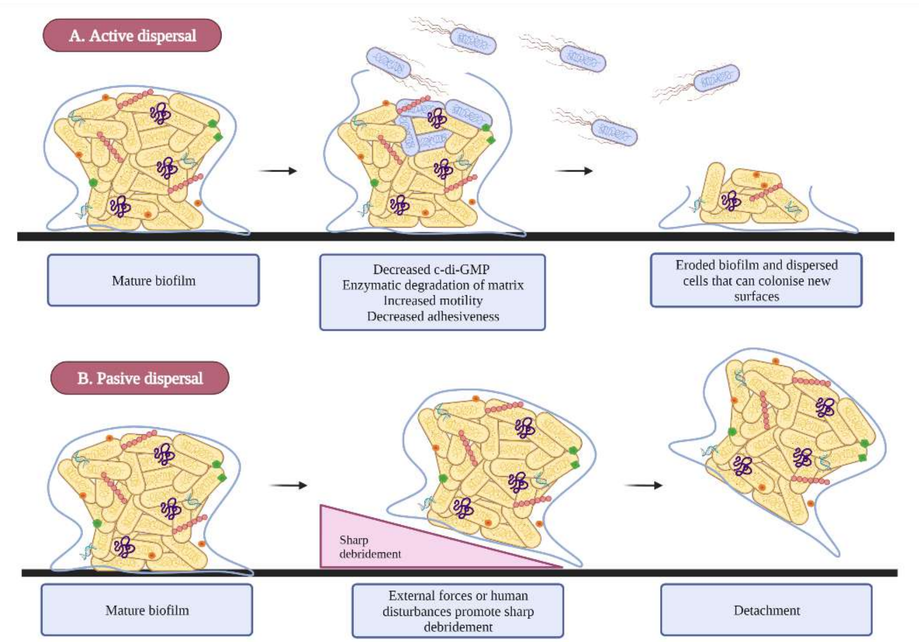


Figure 3. Dispersal process. A. Active dispersal. **B.** Passive dispersal. This figure was created with BioRender.com.

1.3. Biofilms and their role in pathogenesis

Bacterial ability to adhere to surfaces and promote the formation of biofilms is an essential feature of pathogenicity. Once bacteria have colonised

the surface and the biofilm has matured the bacteria from biofilms can disperse and spread throughout the organism. It is estimated that 65% to 80% of human infections involve the development of biofilms (33). These include tissue-related infections such as chronic wounds, chronic otitis, dental plaque, etc., as well as infections associated with indwelling devices such as prosthetic heart valves, cardiac pacemakers, orthopaedic or intrauterine devices, intravascular catheters, renal dialysis shunts, endotracheal tubes, and contact lenses (Figure 4) (1,34,35).

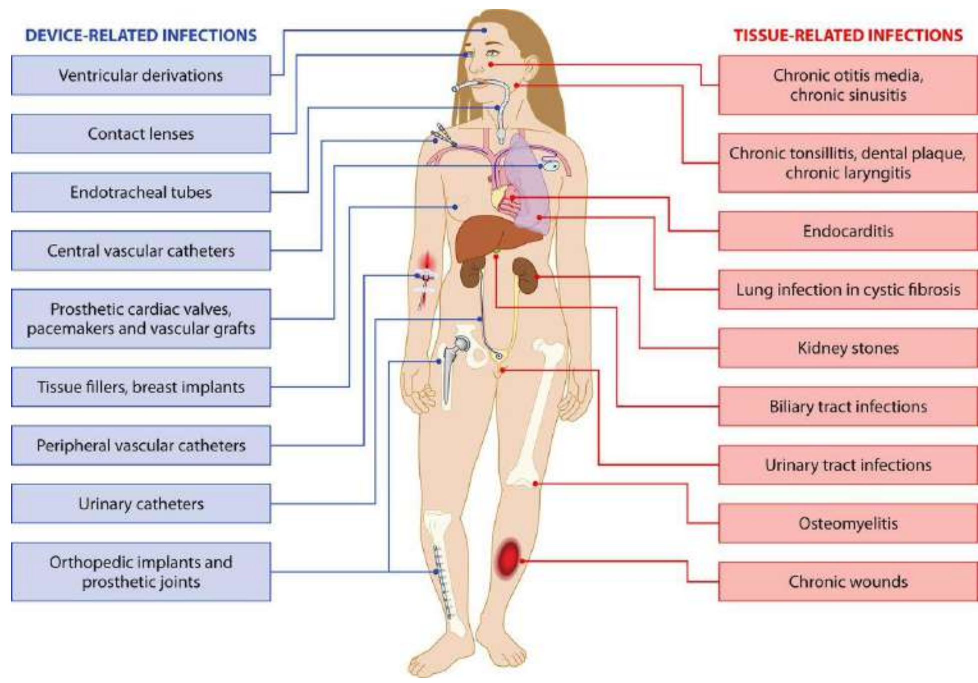


Figure 4. Biofilm-related infections. (Adapted from 34: p.512).

The most common healthcare-associated infections are related to invasive devices, particularly central vascular catheters, urinary catheters, and ventilators. Thus, according to a report from the USA National Nosocomial Infection Surveillance System, three primary infections accounted for 68% of all reported infections, with nosocomial pneumonia being the most common, followed by urinary tract infections (UTIs), and

bloodstream infections (BSIs). Among these, 83% were ventilator-associated pneumonia (VAP), 97% were catheter-associated UTIs (CAUTIs), and 87% of BSIs were bloodstream infections from central venous catheters (36).

1.3.1. Ventilator-associated pneumonia (VAP)

VAP is the most common pulmonary infection in ventilated patients. The intubation procedure bypasses the host's natural defences by suppressing the cough reflex and mucociliary clearance, allowing bacteria from the host's nasopharyngeal microbiota or the external environment access to the lungs (37). Therefore, endotracheal tubes are ideal for bacterial attachment and serve as reservoirs for pathogens that form biofilms (38).

It has been demonstrated that bacteria can grow in mucus secretions, and Landry *et al.* reported that the interaction between mucus mucins and bacterial adhesins promotes biofilm formation (39). Many of these biofilms cause pneumonia when they are in the dispersal stage, suggesting that VAP is independent of the duration of intubation (38).

Seventy percent of VAPs are caused by biofilms formed in the endotracheal tube, which are covered with bacterial lipopolysaccharides (LPS) and extensive bacterial growth (37,40). Moreover, VAP is a major cause of sepsis in the intensive care unit (ICU) and is associated with a high mortality rate. The incidence of bacteraemia and sepsis originating from the upper respiratory tract ranges from 12% to 18% (37).

Microorganisms associated with VAP vary according to geographical area, patient risk factors, length of stay in the ICU, and other features. For example, a study conducted in Poland found *Enterobacterales* (32.6%), non-fermenting Gram-negative bacteria (27.6%), Gram-positive cocci (19.3%), and other pathogens (20.4%) to be associated with VAP (41). Similarly, from 2009 to 2012, the SENTRY Antimicrobial Surveillance Program reported that Gram-negative bacteria were the most frequently isolated organisms related to VAP in the United States and Europe (61.5%/76.1%, respectively), with *P. aeruginosa* being the most common Gram-negative bacteria in both areas.

Among Gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus pneumoniae* were also found in high percentages (42).

1.3.2. Bloodstream infections (BSIs) associated with intravenous catheters

Vascular access devices are mainly used to administer fluids, blood products, medications, nutritional solutions, and haemodynamic monitoring, making it an easy route for BSIs. Factors such as the duration of catheterisation, the catheter material and its mishandling, increase the risk of BSIs (43).

Pathogenesis of catheter-related bloodstream infections (CRBSIs) usually begins when bacteria, mainly from the skin, come in contact with a catheter and then adhere to it forming a biofilm. Then, local infection develops affecting the intravascular catheter. The biofilm spreads from the catheter and the dispersed bacteria contact the bloodstream directly, leading to sepsis (44). Bacteria can also spread to distant body sites, causing local infections such as endocarditis, pneumonia, UTI, meningitis, osteomyelitis, and prosthetic infections, among others (33).

For diagnostic purposes, BSIs are considered catheter-associated if the pathogen isolated from the catheter tip is the same as that isolated in the peripheral blood culture (37).

Interestingly, some studies using scanning (SEM) and transmission electron microscopy (TEM) have shown that virtually all central venous catheters are colonised by microorganisms embedded in a biofilm matrix (45). The microorganisms isolated from catheters are usually Gram-positive bacteria, such as coagulase-negative *Staphylococcus*, *S. aureus*, *Enterococcus faecalis,* and *Streptococcus* spp. Among Gram-negative bacteria, *Pseudomonas aeruginosa*, *K. pneumoniae*, *E. coli*, *Enterobacter* spp., *Acinetobacter baumannii*, and *Proteus mirabilis* are the most frequently found (43). However, the type of

fluid administered via catheters may influence microbial growth. For instance, Gram-positive bacteria, such as *Staphylococcus epidermidis* or *S. aureus,* have greater difficulty growing in intravenous fluids than Gram-negative bacteria, such as *P. aeruginosa, Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *and Pantoea* spp. (45).

1.3.3. Catheter-associated urinary tract infections (CAUTIs)

CAUTIs are one of the most common healthcare-acquired infections. Although they do not have a high mortality rate, they represent a significant burden to the healthcare system, increasing morbidity and treatment costs (46,47).

Urinary catheters provide the ideal environment for adhesion and colonisation by uropathogens, mainly from the periurethral area. Biofilm formation begins in both the inner and outer surfaces of the catheter after catheter insetion (47). Then, bacteria can detach from the catheter and ascend between the mucosa and the catheter into the bladder, leading to bacteriuria. Otherwise, the bacteria may ascend through contamination of the drainage bag (37). Usually a single species is identified after catheter insertion, including *E. coli*, other *Enterobacterales*, *Enterococcus* spp., coagulase-negative *Staphylococcus*, *P. aeruginosa,* and *Candida* spp. (43,47). However, a mature polymicrobial biofilm may develop if the catheter remains in place for a long time (44).

1.4. Immune evasion and antibiotic tolerance in biofilms

One of the major concerns in the medical field is the difficulty of biofilm eradication because bacteria in biofilms can evade the immune system by reducing both leukocyte and antibody-mediated phagocytosis. In addition, compared to their planktonic analogues, these bacteria can be up to 1,000-fold more tolerant to antimicrobial agents (4,8,23,35,48–50), allowing infections to become chronic despite antibiotic treatment (51). Although several mechanisms have been associated with antimicrobial tolerance in biofilms, the most important are shown in Figure 5 and include low

antimicrobial penetration, reduced growth rates and stress responses, persister cells, efflux pumps, and horizontal gene transfer (HGT).

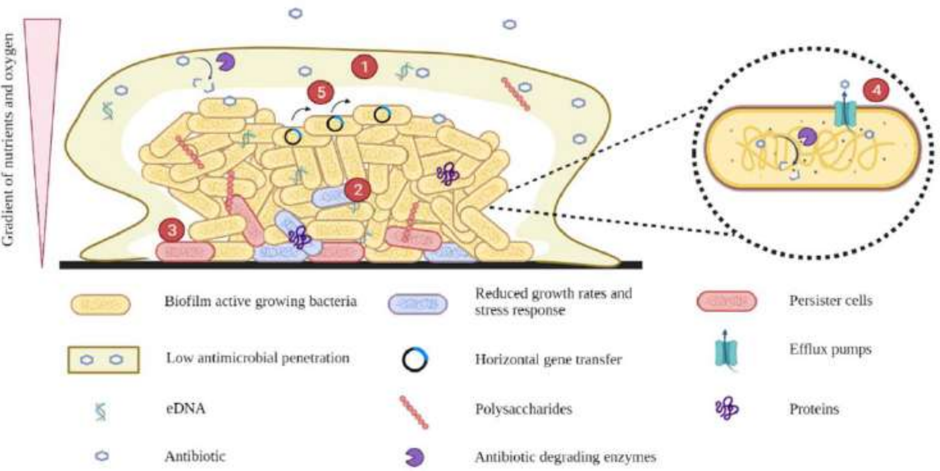


Figure 5. Mechanisms of antimicrobial tolerance in biofilms. This figure was created with BioRender.com. **1.** Low antimicrobial penetration. **2.** Reduced growth rates and stress responses. **3.** Persister cells. **4.** Efflux pumps. **5.** Horizontal gene transfer.

1.4.1. Low antimicrobial penetration

Biofilm matrix acts as a physical barrier and plays an essential role in limiting and retarding penetration of antimicrobial agents into the cells embedded in the biofilm. In addition, structural contents of the matrix, such as charged polysaccharides and eDNA, can bind various molecules inhibiting the diffusion of antimicrobial agents, chelating cations, and suppressing immune response (23,52,53). Some antibiotic-degrading enzymes in the matrix (lyases, transferases, hydrolases, and redox enzymes) can induce antimicrobial resistance by cleaving the chemical bonds that allow the enzymes to function properly or by inhibiting the binding of antibiotics to their targets (52). Both mechanisms, the delay of penetration and the degradation of antimicrobials, have a synergistic effect that confers adequate antimicrobial tolerance to the biofilm (54). Reduction in antibiotic penetration

allows the bacteria to develop an adaptive response that can lead to reduced sensitivity to antimicrobials (53).

1.4.2. Reduced growth rates and stress responses

Within biofilms, bacteria in deeper layers show lower metabolic activity, growth, and division due to the oxygen and nutrient gradient present along the biofilm. This gradient results from the consumption of available nutrients and oxygen by the cells closest to the surface, avoiding the spread of nutrients to the centre of the biofilm (53). As a result, phenotypic diversity is observed within the biofilm, promoting differential gene expression and leading to antibiotic tolerance by regulating genes involved in deoxyribonucleic acid (DNA) repair, lipid biosynthesis, toxin efflux, and ion sequestration (52).

In addition, temperature fluctuations, changes in pH or osmolarity, and high cell density, activate the general stress response system regulated by the σ-factor RpoS, which protects cells from the environment. Adaptive stress responses influence antimicrobial susceptibility because they affect the cellular components and processes targeted by antibiotics (53). Biofilm bacteria are more tolerant to antibiotics since these are less effective against metabolically inactive or slow-growing cells (23,52,55,56).

1.4.3. Persister cells

A specialised bacterial phenotype is also found within biofilms, differing from others in its growth and sensitivity to antimicrobial agents. Bacteria with this phenotype are called persister cells and are defined as dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics. They develop under stress conditions and show growth rates close to zero or extremely slow. They regulate the toxin-antitoxin system and upregulate phosphate metabolism, enhancing anti-oxidative and DNA repair systems, evading the immune system, and surviving antimicrobial agents designed to act on dividing cells (52). Moreover, persister cells can be reactivated, causing infection once the

selective pressure from antibiotics disappears (23,52). High concentrations of persister cells are observed in chronic UTIs and in the lungs of patients with cystic fibrosis (53).

1.4.4. Efflux pumps

Efflux pumps are membrane proteins responsible for exporting toxic substances, including antibiotics, from inside to outside bacteria (57). Although they are also found in planktonic bacteria, their overexpression in biofilms can lead to the phenotype of multidrug resistance (MDR).

There are five classes of bacterial efflux pumps: the major facilitator superfamily (MFS), the small MDR family, the ATP-binding cassette (ABC) family, the resistance nodulation-division (RND) family, and the multidrug and toxic extrusion (MATE) family (Figure 6) (57).

Efflux pumps have been associated with biofilm formation. For instance, it has been reported that some efflux pumps of the MDR family contribute to the formation of biofilms by helping bacteria evade attack from different antibiotics (53). In addition, the genes encoding the AcrAB-TolC efflux pump, which belongs to the RND family, were found to be upregulated in *E. coli* biofilms after exposure to several antibiotics (58). On the other hand, *E. coli* mutants with altered efflux pump genes have been reported to have a lower ability to form biofilms (59). Surprisingly, deletion of the *tolC* gene from enteroaggregative *E. coli* (EAEC) showed low adhesion and biofilm formation associated with reduced expression of aggregative fimbriae (58). In addition, deletion of the *emrD, emrE, emrK, acrD, acrE* or *mdtE* genes, which encode proton motive force pumps in *E. coli*, resulted in a lower biofilm-forming capacity than the wild-type (wt) strain (59).

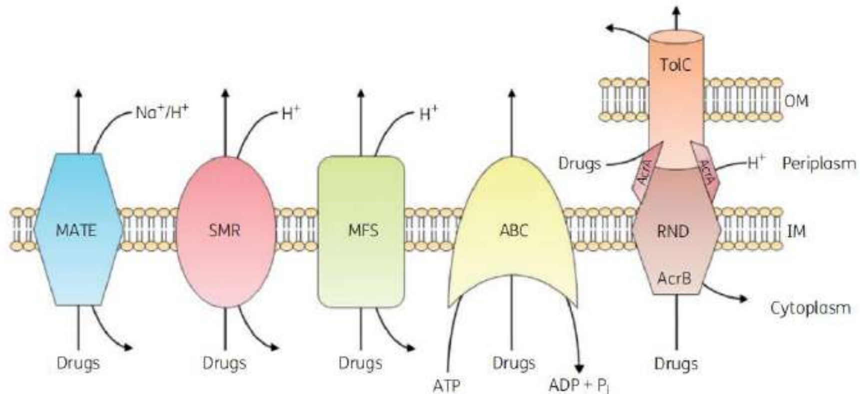


Figure 6. Superfamilies of efflux pumps found in bacteria. MATE, multidrug and toxic extrusion family; SMR, small multidrug resistance family; MFS, major facilitator superfamily; ABC, ATP-binding cassette family; RND, resistance nodulation-division family (Adapted from 57: p.2007).

1.4.5. Horizontal gene transfer (HGT)

Due to the high population density in biofilms, interactions between cells increase, favouring HGT.

Antimicrobial resistance genes (ARGs) are contained on mobile genetic elements (MGEs) that can easily be transferred between cells and promote antimicrobial resistance. Although this mechanism is also observed in planktonic cells, it occurs significantly in biofilms. Some researchers have demonstrated that conjugation is more effective in biofilms than in planktonic cells because bacteria harbouring conjugative plasmids and susceptible bacteria are close and in continuous contact with each other. In addition, the bacteria can pick up free DNA from the matrix. Therefore, biofilms play an important role in the spread of ARGs and can be considered a reservoir for genetic diversity (60).

2. Polymicrobial biofilms

In nature, biofilms are usually formed by several species that interact with each other, influencing the development of the community positively or negatively (1,61).

2.1. Interspecies interactions

Interactions between species involve communication (typically via QS) and metabolic cooperation or competition (62).

In cooperation or synergism (Figure 7A), the species involved work together by increasing biofilm formation and, consequently, their tolerance to antibiotics and virulence. They even improve the degradation of organic compounds (61,63–66).

In the case of competition or antagonism (Figure 7B), the resulting interaction gives one of the involved species an advantage based on nutrient competition or inhibition of their counterparts (61,63–66).

Nevertheless, if neither synergism nor antagonism can be detected between the species involved, the interaction is considered **neutral** (Figure 7C).

2.1.1. Cooperation

Several mechanisms are involved in the cooperation of species in biofilms. These include the secretion of compounds such as nutrient chelators, digestive enzymes, surface adhesins, wetting agents, structural polymers, and signalling molecules (67). The autoinducer AI-2 is the most important for intra-species communication, as it is found in both Gram-negative and Gram-positive bacteria (61).

Studies on cooperative behaviour in biofilms have been published. In polymicrobial biofilms formed by *E. faecalis* and *E. coli*, the export of L-ornithine by *E. faecalis* stimulates the production of chelating agents such as enterobactin by *E. coli*, overcoming iron limitation and promoting adequate growth (68). In the case of the oral biofilm formed by *Streptococcus gordonii* and *Porphyromonas gingivalis*, the exopolysaccharide produced by *S. gordonii* can act as a receptor for a surface adhesin expressed by *P. gingivalis,* facilitating their colonisation on the tooth surface (69). On the other hand, in the polymicrobial biofilm formed by *Pseudomonas putida* and *Acinetobacter* sp., both species can use benzyl alcohol as their sole carbon source. Although a competitive relationship for the same carbon source is conceivable, *Acinetobacter* growing on benzyl alcohol produces benzoate, which is metabolised by *P. putida*, allowing cooperative interaction between the two (70).

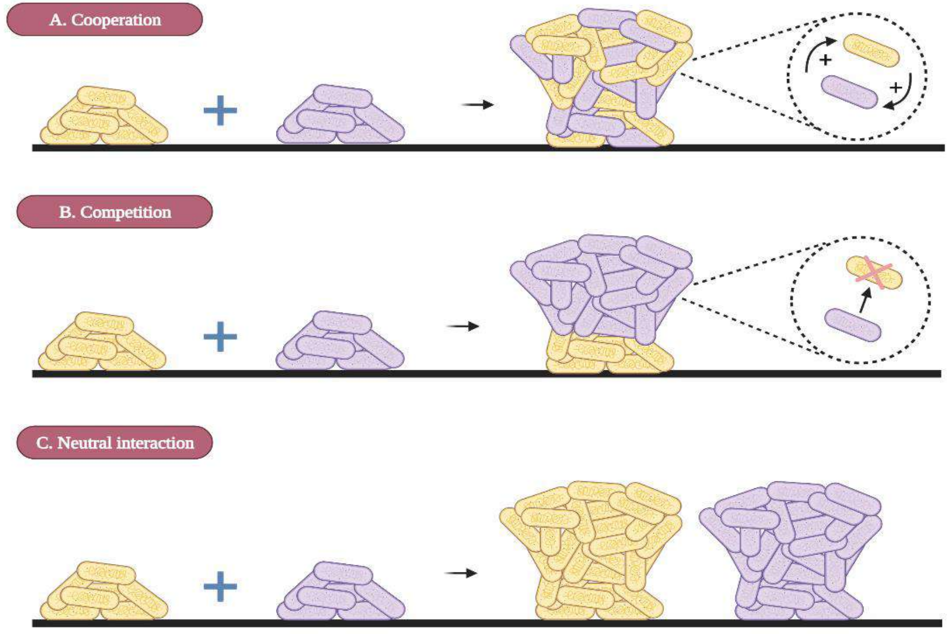


Figure 7. Interspecies interactions in biofilms. A. Cooperation. **B.** Competition. **C.** Neutral interaction. This figure was created with BioRender.com.

Another mechanism of cooperation in mixed biofilms occurs when one member of the community creates conditions that promote the survival of the other members in hostile environments. For example, anaerobic bacteria can survive under aerobic conditions when grown in the presence of aerobic bacteria, as aerobic bacteria consume oxygen and promote an ideal anaerobic environment in the deeper layers of the biofilm (61). Similarly, a synergistic interaction is observed between the two pathogens most commonly found in the lungs of cystic fibrosis patients, *S. aureus* and *P. aeruginosa*. It appears that the exoproduct of *P. aeruginosa*, 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), increases the aminoglycoside resistance of *S. aureus*, leading to the selection of aminoglycoside-resistant small-colony variants of *S. aureus* that are difficult to detect and treat (71).

2.1.2. Competition

Resources become limited within a crowded polymicrobial biofilm, and thus, community members tend to compete with each other. This competition can occur at any stage of the biofilm life cycle: initial or irreversible adhesion, maturation or dispersal (72). Thus, one species inhibits the growth of the other by producing harmful compounds (including antibiotics or toxins), consuming essential nutrients, restricting space, and displacing or suffocating its neighbours (61,67).

The oral cavity is a niche in which competitive interactions are well observed and studied. *Enterococcus faecium*, for example, produces an inhibitory protein that restricts the biofilm formation of three oral pathogens, *Streptococcus sobrinus*, *Streptococcus sanguinis,* and *Streptococcus mutans* (73). In the case of polymicrobial biofilms formed by *S. sanguinis*, *S. gordonii*, and *S. mutans*, the two first species inhibit the growth of *S. mutans* by the production of hydrogen peroxide (74).

An example of competition by spatial distribution is observed within a mixed-species biofilm formed by *P. aeruginosa* and *Agrobacterium tumefaciens*. In this case, *P. aeruginosa* dominates the biofilm by covering the

A. tumefaciens cells adhered to the surface, a phenomenon termed 'blanketing' mediated by QS and motility via pili and flagella (75).

The best-studied compounds in competitive interactions are bacteriocins. Bacteriocins are proteinaceous or peptidic antimicrobial substances produced by bacteria to inhibit or kill other related or unrelated strains without harming the producing bacteria due to specific immune proteins (76). Among these, the antibiofilm effect of the bacteriocin produced by *Lactobacillus brevis* DF01 on *E. coli* and *Salmonella enterica* serovar Typhimurium (77) and the effect of bacteriocin EntV produced by *E. faecalis* on reducing the virulence and biofilm formation of *Candida albicans* by inhibiting hyphal morphogenesis (78) have been well-studied. In contrast to Hoffman *et al.*, who found a cooperative interaction between *S. aureus* and *P. aeruginosa* (71), Korgaonkar *et al.* found a competitive interaction between the two pathogens due to the toxin pyocyanin produced by *P. aeruginosa* in response to N-acetylglucosamine and peptidoglycan from the cell walls of *S. aureus* (79).

2.2. Polymicrobial biofilms formed by *K. pneumoniae*

K. pneumoniae is an important opportunistic pathogen commonly associated with UTIs, including CAUTIs (80,81). Moreover, *K. pneumoniae* belongs to the ESKAPE group, which consists of antibiotic-resistant bacteria (*E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa and Enterobacter* spp.) that can escape the biocidal effects of antibiotics and represent new paradigms in pathogenesis, transmission, and resistance (82).

The first study of mixed microbial populations with *K. pneumoniae* dates from 1991. Siebel *et al.* demonstrated the ability of *K. pneumoniae* to form a polymicrobial biofilm with *P. aeruginosa.* The results showed that although the specific growth rate of *K. pneumoniae* was five times higher than that of *P. aeruginosa*, *K. pneumoniae* did not dominate the population in the biofilm. Consequently, no interaction between these two species was observed (83).

In 1998, a plasmid encoding the green fluorescent protein of *Aequorea victoria* was introduced into a biofilm-forming strain of *Enterobacter*

agglomerans. This *E. agglomerans* strain was co-cultured with *K. pneumoniae* in biofilms. Microscopic analysis showed that *E. agglomerans* interacts with *K. pneumoniae* and forms more successful biofilms in combination than when cultured in a single-species biofilm, suggesting that the interactions involve surface-associated molecules. This co-existence leads to improved biofilm formation and increased resistance to disinfection (84).

In 2007, 106 samples of biofilms developed on urinary catheters were analysed, finding that *Providencia stuartii* and *K. pneumoniae* strains were frequently associated with *P. mirabilis*; while *E. coli, Morganella morganii* or *Enterobacter cloacae* strains were rarely associated with this pathogen (85).

In a study conducted in 2014, *K. pneumoniae* displayed the ability to form a mixed polymicrobial biofilm with *P. aeruginosa* and *Pseudomonas protegens*. A fluorescent tag labelling each species determined their abundance and spatial location within the biofilm. The development of the polymicrobial biofilm was delayed by 1-2 days compared to the monomicrobial biofilm. However, the polymicrobial biofilm was more resistant to sodium dodecyl sulfate (SDS) and tobramycin than the single-species biofilms. These results suggested that cooperative interactions, especially resource sharing, were predominant (86). In the same year, it was demonstrated that *K. pneumoniae* could form multispecies biofilms with *C. albicans* in urinary catheters (81).

In the present doctoral thesis, the polymicrobial biofilm of *K. pneumoniae* and *E. faecalis* was investigated (Paper 1).

3. Targets and molecules to control biofilms

The increase in antibiotic resistance, combined with the ability of some bacteria to form biofilms, is a threat to public health and biomedical research worldwide. In many cases, current antibiotic therapy can only reduce but not completely eradicate biofilms, as the high concentrations of antibiotics required for complete eradication can become toxic to the patient (87).

In section 1.2.2, it was already mentioned that the biofilm matrix provides protection from adverse environmental conditions and allows bacteria to be tolerant of antimicrobial agents. Therefore, there is an urgent need to develop new therapeutic strategies to counteract this problem.

Some of the essential strategies to achieve this problem are as follows:

(i) Identify new targets: This strategy typically uses random transposon insertion, a high-throughput genetic manipulation tool that enables the random mutation of genes at the genome level, mainly through loss-of-function screening. This tool creates random mutant libraries in which genetic factors responsible for the expression of the phenotypes of interest are identified. These genes can then be considered new targets for testing the effect of potential antibiofilm molecules (88,89).

This thesis presents the discovery of a new gene involved in biofilm formation in *E. coli* using this technique (Paper 2).

(ii) Design and screening of new molecules: Several molecules that attempt to eradicate or inhibit biofilms are being investigated. These compounds act at different stages of biofilm formation and include inhibition of adhesion and QS, dispersal strategies, and disruption of the EPS matrix. Compounds with higher antibiofilm activity are discussed in this section and summarised in Table 1.

This thesis also investigates the antimicrobial and antibiofilm activity of a fruit extract against several Gram-positive and Gram-negative bacteria (Manuscript 1).

3.1. Adhesion inhibition

Inhibiting adhesion, the first stage of biofilm formation, is an excellent preventive strategy to combat biofilms. Some molecules inhibit the biosynthesis of fimbriae, surface proteins, VFGs, and other bacterial structures involved in this step. Among these, the following compounds are particularly noteworthy:

3.1.1. Ethyl acetate fraction of Cocculus trilobus

Ethyl acetate extracts of the medicinal plant *Cocculus trilobus* inhibit the bacterial protein sortase, a membrane enzyme of Gram-positive bacteria that cleaves surface proteins involved in adhesion into peptidoglycan. Thus, bacterial adhesion is prevented if sortase is inhibited (27,90).

3.1.2. Cranberry polyphenols

Cranberries are a rich source of polyphenols. Polyphenols inhibit biofilm formation by preventing cariogenic bacteria from adhering to and colonising host tissues. They disrupt the virulence factors involved in adhesion, acidogenicity, and glucan synthesis without affecting microbial viability (27,91). A study by Sánchez *et al.* concluded that cranberry polyphenols interfered with the biofilm formation of *P. gingivalis, Aggregatibacter actinomycetemcomitans*, and *F. nucleatum* by affecting bacterial adhesion in the first 6 h of biofilm development (92). Thus, they can be used to treat oral infections such as periodontitis and dental caries.

3.1.3. Ginkgo biloba extract

Ginkgo biloba extract and ginkgolic acid significantly inhibit the formation of enterohaemorrhagic *E. coli* (EHEC) O157:H7 biofilms without affecting the growth of commensal *E. coli*. It represses curli genes and reduces

fimbriae production, thereby interrupting adhesion and biofilm formation (27,93). This extract also has an antibiofilm effect against *Listeria* spp. and *Salmonella* spp. (94), and against methicillin-resistant *S. aureus* (MRSA) by limiting the expression of *sarA* (staphylococcal accessory regulator A) and *sigB* (transcription factor) genes, which regulate the secretion of virulence factors and biofilm formation in this species (95).

3.1.4. Phloretin

The apple flavonoid phloretin (Figure 8) inhibits EHEC O157:H7 biofilms by reducing fimbrial production without affecting commensal bacteria. Phloretin represses toxin genes (*hlyE* and *stx2*), AI-2 importer genes (*lsrACDBF*), and curli genes (*csgA* and *csgB)*, also preventing bacteria from attaching to human epithelial cells. This molecule acts not only as an antibiofilm agent but also as an anti-inflammatory substance (27,96). Furthermore, phloretin has excellent antibiofilm activity against *S. aureus* strains SA1199B and RN4220, inhibiting up to 70% of biofilms and acting on efflux proteins (97).

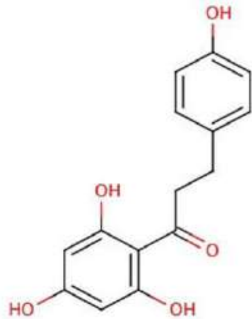


Figure 8. Phloretin structure

3.2. Quorum sensing (QS) inhibitors

As explained in section 1.2.2, QS is a signalling process that enables cell-to-cell communication to coordinate the expression of specific genes and biofilm maturation. QS inhibitors are a promising therapeutic alternative against biofilms. They disrupt the signalling pathway used for intra- and interspecies communication, altering the expression of several virulence factors, and counteracting bacterial pathogenicity (98). Different agents are considered QS inhibitors and act against biofilm formation in Gram-negative bacteria through three primary strategies, including blocking the biosynthesis of AHL molecules, inactivating or degrading AHL molecules, and interfering with the signal receptor through antagonists (Figure 9) (99).

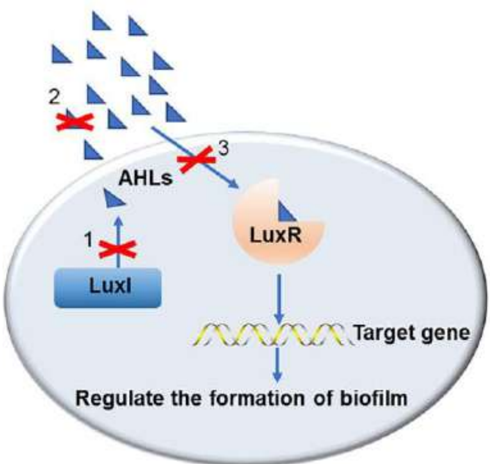


Figure 9. The mechanisms of QS inhibitory agents in controlling bacterial biofilm formation. 1. Blockage of the biosynthesis of AHL molecules. **2.** Inactivation or degradation of AHL molecules. **3.** Interference with the signal receptor using antagonists. (Adapted from 99: p.4).

These inhibitors include furanone, quercetin, curcumin, ginseng extract, and garlic extract, among others (87,100,101), but only the most studied are briefly explained here.

3.2.1. Furanone

Furanone is a secondary metabolite derived from the Australian macroalga *Delisea pulchra* that suppresses QS and bacterial motility (Figure 10). Furanone acts as an antagonistic molecule by competing with AHL for the LuxR receptor site, interrupting bacterial QS signalling. It also disrupts the alternative AI-2 signalling system in Gram-negative and Gram-positive bacteria (87,100). In one study, this compound reduced the formation of biofilms by *Serratia liquefaciens* on polystyrene, had a synergistic effect when combined with the phenolic extract of Pitanga (*Eugenia uniflora*) or kanamycin, and completely inhibited the growth of *S. liquefaciens* (102). Similarly, some studies have shown that synthetic furanone derivatives 56

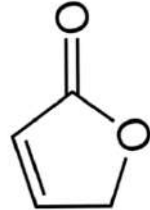


Figure 10. Furanone structure

and C-30 inhibit *P. aeruginosa* biofilm formation by impairing QS in planktonic cultures and mature biofilms (103,104).

3.2.2. Quercetin

Quercetin is a plant-derived flavonoid found in many grains, vegetables and fruits (Figure 11) (97). It inhibits biofilm formation by reducing the expression of the proteins LasI, LasR, RhlI and RhlR, which are involved in QS of *P. aeruginosa*, reducing adhesion and EPS production, inhibiting efflux pumps and blocking nucleic acid synthesis (105). A concentration of 80 µg/mL quercetin reduces QS-mediated processes, including biofilm formation, violacein production, motility, and EPS production in many foodborne pathogens (106). Some studies have reported an antibiofilm effect of quercetin against *E. faecalis, S. aureus, S. mutans, E. coli,* and *P. aeruginosa* (27,87,105).

Figure 11. Quercetin structure

3.2.3. Curcumin

Curcumin (Figure 12) is a phytochemical produced by plants of the species *Curcuma longa* that inhibits bacterial virulence factors and has an antibiofilm effect by inhibiting the AHL synthase required for QS. In addition, curcumin also prevents bacterial adhesion to host cells (87,107).

Figure 12. Curcumin structure

The study conducted by Raorane *et al.* showed that curcumin inhibited the surface motility and biofilm formation of *A. baumannii*. It also had antibiofilm activity against *C. albicans* mono-species biofilms and polymicrobial biofilms formed by *C. albicans* and *A. baumannii*. Molecular docking analysis indicated that curcumin interacts with the biofilm response

regulator BfmR of *A. baumannii* (108). Likewise, curcumin inhibits *P. aeruginosa* PAO1 virulence factors, including biofilm formation, pyocyanin biosynthesis, elastase/protease activity, and the production of homoserine lactone (109).

3.3. Degradation of extracellular polymeric substances

Degradation of the structural components of biofilms is considered a potential biofilm control strategy aimed to increase the penetration of common antimicrobial agents. Some of the most studied agents are as follows:

3.3.1. Glycoside hydrolases

Glycoside hydrolases such as cellulase, mannosidases, alginate lyase, α-amylase and dispersin B (DspB) act directly against extracellular proteins. DspB, produced by *A. actinomycetemcomitans*, is involved in the degradation of poly-β-(1,6)-N-acetylglucosamine (PNAG) by hydrolysis of the β-1,6 glycosidic linkages (101,110). DspB and α-amylase are also involved in the degradation of eDNA (111). DspB has potent activity in removing *S. epidermidis* biofilms. Furthermore, pre-coating surfaces with the DspB enzyme prevents the formation of *S. epidermidis* biofilms (112).

3.3.2. Proteases

Some proteases can destroy the integrity of biofilms by degrading extracellular proteins and the biofilm matrix. Among them, we found proteinase K, trypsin, chymotrypsin, serratiopeptidase, carboxypeptidase A, alginate lyase, actinidin (Figure 13), and papain (Figure 14), among others (101,110).

Trypsin (from the porcine pancreas), papain (from *Carica papaya*), and actinidin (from kiwi fruit) have antibiofilm activity on *Actinomyces* monospecies biofilms and multispecies oral biofilms by digesting fimbriae (113). Similarly, the peptidase M16, secreted by *Microbacterium* sp. SKS10 degrades *S. aureus* biofilms at lower concentrations than proteases such as

papain, trypsin, and α-amylase. Furthermore, this peptidase showed synergistic activity that increased the penetration of this antibiotic into the biofilm when combined with kanamycin (114).

Figure 13. Actinidin structure

Figure 14. Papain structure

3.3.3. Phages

Specific phages and their polysaccharide-degrading enzymes can destroy and eliminate biofilms (101), and due to their species specificity, they usually do not affect the host-microbiota (115). Ahiwale *et al.* confirmed that the T7-like lytic phage could degrade and prevent biofilms formed by clinical *P. aeruginosa* isolates (116). Furthermore, phage therapy has also been combined with antimicrobials to allow deeper penetration of these into biofilms (117). For instance, Verma *et al.* treated mature *K. pneumoniae* biofilms with the lytic bacteriophage KPO1K2 combined with ciprofloxacin, and found a more significant reduction in the number of bacterial cells than in biofilms treated with ciprofloxacin alone (118).

3.3.4. Myricetin

The flavonoid myricetin (Figure 15) inhibits the expression of genes associated with EPS synthesis and the expression of *gtfBC* genes essential for cariogenic biofilms of *S. mutans* (119). Myricetin also inhibits curli-dependent biofilm formation of *E. coli* by inhibiting the cellular functions of DnaK

Figure 15. Myricetin structure

(120). The study by Matilla-Cuenca *et al.* showed that myricetin has an inhibitory effect against biofilms of several *S. aureus* strains and other staphylococcal species expressing the biofilm-associated protein (Bap) by inhibiting the Bap amyloid-like aggregates. In addition, they found that myricetin reduced the colonisation of catheters by *S. aureus* in a subcutaneous catheter model (121).

3.4. Biofilms dispersion

Some molecules can cause biofilm disruption by acting as dispersal signals when microorganisms recognise them in the biofilm (122). Among these, the following can be found:

3.4.1. Sequestration molecules

The most studied sequestration molecules are those that inhibit the c-di-GMP signalling system. c-di-GMP is a conserved biofilm mediator that plays a crucial role in regulating biofilm formation and maintenance. High levels of c-di-GMP promote biofilm formation, and low levels of c-di-GMP promote dispersion (Figure 16) (123,124). Antagonists of diguanylate cyclase, the enzyme synthesising c-di-GMP, and molecules that bind c-di-GMP are potential dispersal agents.

In this sense, BdcA is a protein produced by *E. coli* that binds free c-di-GMP and indirectly inhibits the role of c-di-GMP in biofilm maintenance. Another sequestration molecule is cis-2-decenoic acid, also known as diffusible signalling factor and initially produced by *P. aeruginosa*, which exhibits dispersal activity against biofilms formed by *P. aeruginosa, E. coli, K. pneumoniae, P. mirabilis, S. pyogenes, Bacillus subtilis, S. aureus, C. albicans, S. enterica,* and *S. mutans* (122).

3.4.2. Antimicrobial peptides

Antimicrobial peptides (AMPs) are a new approach in treating biofilm infections. More than 2,600 peptides with antimicrobial activity have been

isolated from various sources, including animals, plants, fungi, and bacteria (122).

One of the most studied is AMP 1018, a synthetic peptide modified from bactenecin that suppresses the alarmone ((p)ppGpp) signal. Alarmone is a molecule synthesised by bacteria in response to stress and nutrient deficiency that regulates the stringent response. The absence of (p)ppGpp reduces antibiotic tolerance and virulence, impairing biofilm formation. This peptide is effective in mature biofilms of *P. aeruginosa, E. coli, A. baumannii, K. pneumoniae, S. aureus, S. enterica* serovar Typhimurium*, and Burkholderia cenocepacia* (87,101,122).

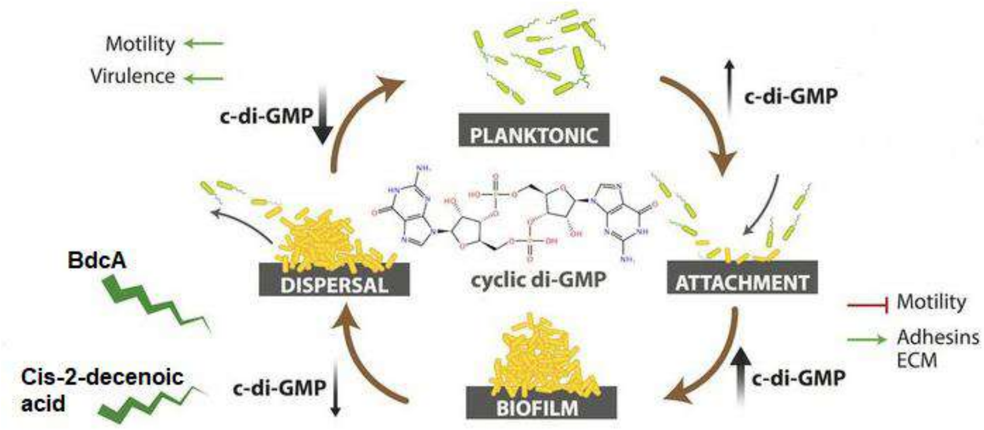


Figure 16. Effects of c-di-GMP on biofilm formation. (Adapted from 124: p.3).

Table 1. Targets and some molecules to control biofilms.

Stage or structure affected	Molecules	Mechanism of action
Adhesion	Extract of *Cocculus trilobus*	Cleaves surface proteins of Gram-positive bacteria
	Cranberry polyphenols	Disrupt virulence factors involved in adhesion, acidogenicity and glucan synthesis
	Ginkgo biloba extracts	Repress curli genes and reduce the production of fimbriae
	Phloretin	Reduces production of fimbriae, curli fibres and toxin genes
Quorum sensing	Furanone	An antagonistic molecule that suppresses QS and bacterial motility
	Quercetin	Reduces the expression of LAsI, LasR, Rh1I and Rh1R involved in QS
	Curcumin	Inhibits the AHL synthase required for QS
EPS matrix	Dispersin B	Degradation of PNAG by hydrolysis of the β-1,6 glycosidic linkages
	Dispersin B and α-amylase	Degradation of eDNA
	Proteinase K, chymotrypsin, serratiopeptidase carboxypeptidase A, alginate lyase	Proteases involved in matrix degradation
	Trypsin, papain, actinidin	Proteases involved in matrix degradation and digestion of fimbriae
	Phages	Their polysaccharide-degrading enzymes can destroy EPS matrix
	Myricetin	Inhibits the expression of genes involved in EPS synthesis
Dispersion	BdcA	Binds free c-di-GMP inhibiting their role in biofilm maintenance
	Cis-2-decenoic acid	Exhibit dispersal activity against biofilms formed by different pathogens
	AMP 1018	Suppresses the alarmone ((p)ppGpp) regulated stringent response

EPS: Extracellular polymeric substances; QS: Quorum sensing; PNAG: Poly-β-(1,6)-N-acetylglucosamine; eDNA: Extracellular deoxyribonucleic acid; c-di-GMP: Cellular bis-(3′-5′)-cyclic dimeric guanosine monophosphate.

4. Virulence determinants

The term virulence is defined as the ability of an organism to infect a susceptible host and cause disease. Some virulence determinants, such as secreted molecules and membrane-associated or cytosolic factors, act individually or together (125,126) and are involved in adherence, evasion of the host immune system, iron uptake, and secretion of toxins. This thesis analyses some of the major virulence determinants of *K. pneumoniae* and *E. coli*. Although both species share several virulence factors, others are unique.

K. pneumoniae is a Gram-negative encapsulated and non-motile bacterium found in the environment and the gastrointestinal tract. It is a common opportunistic pathogen that frequently causes nosocomial infections, including pneumonia, meningitis, BSIs, and UTIs (127,128). Virulence factors such as capsules, lipopolysaccharides (LPS), fimbriae, and siderophores are associated with pathogenesis (Figure 17). The increasing number of hypervirulent *K. pneumoniae* (hvKp) strains worldwide is making this area of research even more critical.

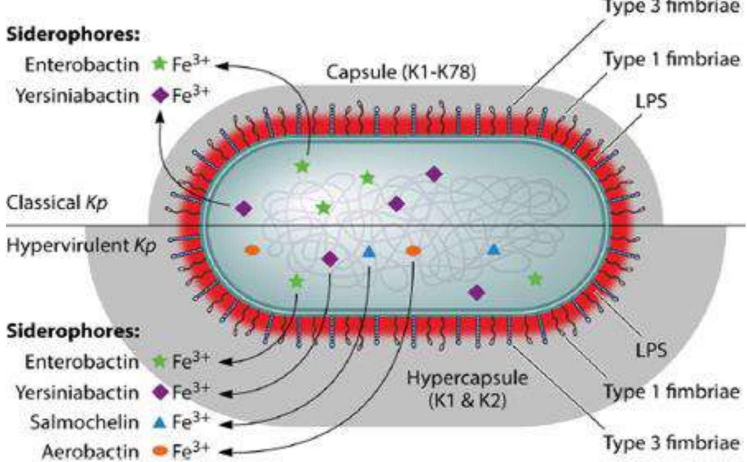


Figure 17. Major virulence factors characterised in *K. pneumoniae*. (Adapted from 128: p.637).

On the other hand, *E. coli* is a Gram-negative rod-shaped, non-spore-forming bacterium. It is usually motile by peritrichous flagella. It is classified as a harmless member of the normal intestinal microbiota of humans (129). Nevertheless, some strains can cause diarrhoea. There are six classic diarrhoeagenic *E. coli* pathotypes: enteropathogenic *E. coli*, Shiga toxin-producing, also referred to as EHEC, EAEC, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, and diffusely adherent *E. coli* (DAEC). In addition, the new pathotype, adherent invasive *E. coli*, has been recognised (130).

Although diarrhoeagenic *E. coli* are of great importance, the present thesis is focused on studying extraintestinal pathogenic *E. coli* (ExPEC) strains. ExPEC strains are frequently implicated in numerous infectious diseases worldwide in the community or healthcare settings, causing high economic and social costs. UTIs are the most common infections caused by ExPEC, followed by BSIs and respiratory tract infections (131). Virulence factors such as fimbriae, siderophores, toxins, among others are involved in the pathogenesis of ExPEC strains (Figure 18).

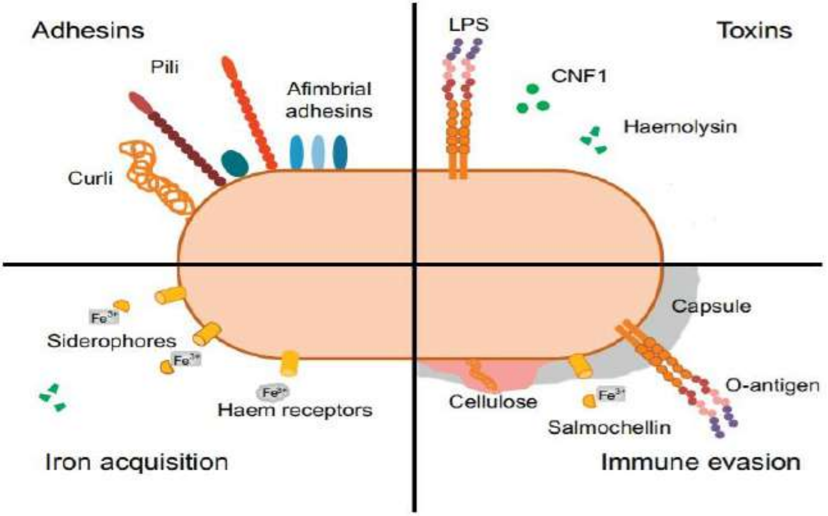


Figure 18. Virulence factors characterised in *E. coli* (Adapted from 133: p.342).

4.1. Virulence factors found in both *K. pneumoniae* and *E. coli* strains

4.1.1. Type 1 fimbriae

Type 1 fimbriae are filamentous organelles expressed on the bacterial cell surface and are involved in bacterial adhesion. They consist mainly of FimA subunits, with the FimH subunit at the tip (Figure 19). They are expressed in 90% of clinical and environmental *K. pneumoniae* isolates and in almost all *Enterobacterales*. The type 1 fimbrial gene cluster of *K. pneumoniae* is homologous to *E. coli*. The function of type 1 fimbriae is the attachment of mannose-containing structures on host cells and the extracellular matrix; hence, type 1 fimbriae are often referred to as "mannose-sensitive" binding fimbriae (128,132).

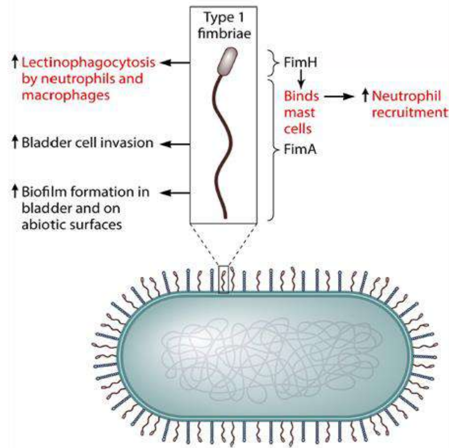


Figure 19. Type 1 fimbriae.
(Adapted from 128: p.640).

Although genes encoding type 1 fimbriae are found in almost all *E. coli* subgroups and not only in urinary pathogenic *E. coli* (UPEC) strains, several studies have shown that they play an essential role in the development of UTIs (132,133). Similarly, these fimbriae of *K. pneumoniae* play a role in invading bladder cells and forming biofilms on the bladder and abiotic surfaces (128).

4.1.2. Siderophores

Siderophores are small iron-binding molecules that chelate iron, an essential element for metabolic processes, growth, and replication of bacteria. Siderophores steal iron from host iron-chelating proteins or capture it from the environment. Enterobactin, aerobactin, yersiniabactin, and salmochelin are detected in *K. pneumoniae* and *E. coli* (Figure 20). Aerobactin has the lowest

affinity for iron, whereas enterobactin has the highest (127,128). In general, all four siderophores can be potentially transferred by plasmids (127).

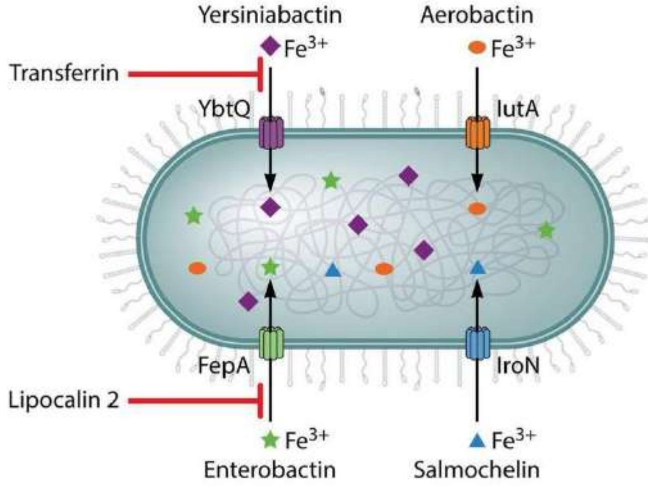


Figure 20. Siderophores. (Adapted from 128: p.642).

4.1.2.1. Enterobactin

Enterobactin was initially identified in *E. coli* and *S. enterica* serovar Typhimurium (134) but has also been found in other *Enterobacterales*, including *K. pneumoniae.* The enterobactin system consists of the siderophore enterobactin encoded by the *ent* gene cluster and its receptor, encoded by the *fepA* gene (127,133). Enterobactin can be inactivated by human lipocalin-2 (Lcn2), a multifunctional protein with multiple antimicrobial properties. Lcn2 is released by various cells, including neutrophils, and triggers inflammatory response (128,135).

4.1.2.2. Aerobactin

Aerobactin was the first siderophore discovered in *Aerobacter aerogenes* (134). Aerobactin is a citrate-hydroxamate siderophore encoded by the *iucABCD* gene cluster, and the *iutA* gene encodes its transporter. These genes can be located on virulence plasmids and, in some cases, on the same plasmid carrying *rmpA*, an enhancer of capsule production in *K. pneumoniae* (128). Most ExPEC strains harbour aerobactin, whereas it is harboured in only

one-third of normal faecal isolates (136). Unlike enterobactin, aerobactin escapes human Lcn2.

4.1.2.3. Salmochelin

Salmochelin molecules were initially found in *Salmonella enterica* (134). Salmochelin is a glucosylated form of enterobactin. The genes encoding salmochelin are located either on the chromosome or on a plasmid containing the *iroBCDEN* gene cluster. IroN, an outer membrane protein with homology to TonB-dependent receptors, mediates iron transport. It acts as an internalisation factor during the invasion of urothelial cells by ExPEC strains (127,128,136). As Lcn2 does not inhibit salmochelin, it can be helpful in bypassing the immune system (133).

4.1.2.4. Yersiniabactin

The phenolate siderophore yersiniabactin was first discovered in *Yersinia enterocolitica* but has been detected in other bacteria (134). This molecule is part of high-pathogenicity island (HPI) of *Yersinia* and it is codified by the *irp* genes. In addition, the *ybt* and *fyu* genes encode transporters, and the *ybtQ* gene encodes the receptor for uptake (127,128). Yersiniabactin cannot acquire iron in the presence of the host protein transferrin (128). However, it evades binding to Lcn2, preventing inflammatory response and promoting bacterial growth and spreading (135). Yersiniabactin also establishes UTI by UPEC and promotes biofilm formation in human urine (134,137).

4.1.3. Capsular polysaccharide (K antigen)

The bacterial capsule is an extracellular structure that covers the outer layer of the cell wall. Its principal function is to protect bacteria from different physical, chemical, and biological stresses. It is involved in biofilm formation and helps Gram-negative bacteria evade phagocytosis, serum killing, and opsonisation. The capsule is composed of long-chain capsular polysaccharides (CPS), linear polymers of repeating carbohydrate subunits that sometimes contain amino acids or lipids (Figure 21) (138,139). The capsule is produced by a Wzy-dependent process for which the synthesis and

export machinery is encoded in a single 10-30 kb region of the genome known as the K locus (140).

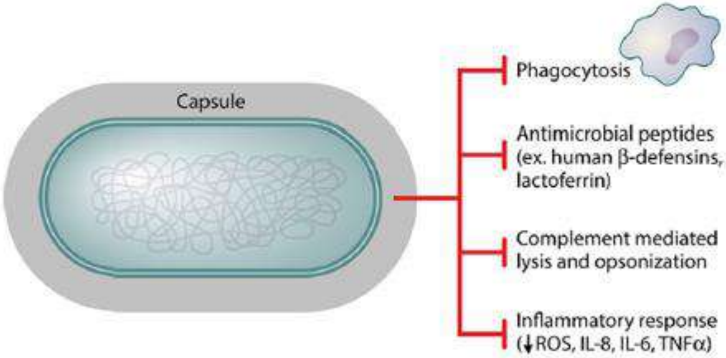


Figure 21. Capsular polysaccharide (K antigen). (Adapted from 128: p.637).

In *E. coli*, more than 80 capsular antigens have been identified, with K1 and K2 being considered the most significant determinants related to ExPEC serum resistance (138).

In *Klebsiella* spp., 79 different capsular phenotypes have been recognised by serological typing, but many isolates are not serotypable (141). Among hvKp, K1, K2, K5, K16, K20, K54, K57, and KN1 have been described, with K1 and K2 being the most common (127). In addition, hvKp strains produce a hyper-capsule, a phenotype known as hypermucoviscous (HMV), consisting of a mucoviscous exopolysaccharide envelope that is more robust than the typical capsule, which increases the pathogenicity of *K. pneumoniae* (128). The production of capsular polysaccharides in *K. pneumoniae* is regulated by the *rmpA* gene, a virulence factor gene localised on a plasmid of hypermucoviscous strains and closely linked to the hvKp phenotype (142). In mouse models, it was demonstrated that acapsular *K. pneumoniae* strains were less virulent than isogenic encapsulated strains (128).

4.1.4. Lipopolysaccharide (LPS) (O antigen)

In Gram-negative bacteria, LPS is one of the most important structural molecules of the outer membrane. LPS consists of three domains: hydrophobic lipid A, core polysaccharide, and long-chain oligosaccharides (O-specific antigen) (Figure 22) (143,144). The O antigen is the outermost component of LPS and is formed by a polymer of repeating oligosaccharide units (145). It is one of the most variable cellular structures due to the variation in sugar composition, resulting in a range of serologically distinct O antigens (144,146).

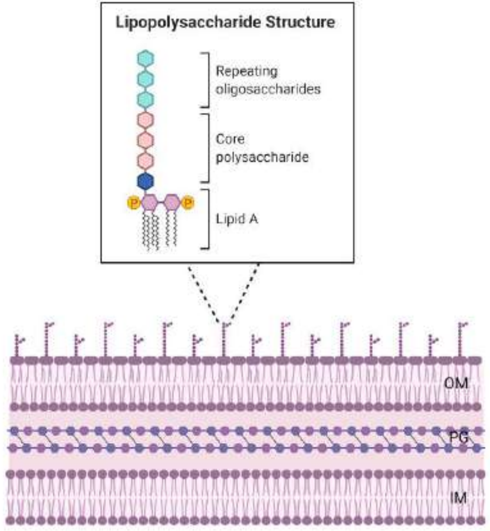


Figure 22. Lipopolysaccharide structure. OM: Outer membrane; PG: Peptidoglycan; IM, Inner membrane. This figure was created with BioRender.com.

LPS has high immunogenicity, activating the innate immune response via TLR-4, a family of immune system receptors found on monocytes, macrophages, dendritic cells, and neutrophils (147). The induction of the immune response subsequently triggers the production of cytokines, chemokines, and interferons (148).

In *K. pneumoniae,* LPS production is controlled by uridine diphosphate galacturonate 4-epimerase, which is encoded by the *uge* gene (149). In

addition, the capsule-associated *wabG* gene, which is involved in the biosynthesis of core LPS (143), and *ycfM*, which encodes the outer membrane lipoprotein, are strongly involved in virulence by promoting infection through resistance to phagocytosis (150). Only nine major O serotypes have been reported in *K. pneumoniae*, with O1, O2, and O3 being responsible for almost 80% of all *Klebsiella* infections (151).

In contrast, *E. coli* comprises 197 O serotypes (including 21 subgroups) encoded in a cluster of 5 to 18 genes located between two housekeeping genes, *galF* and *gnd*. Serotyping based on O antigen variability has become the primary method for classifying *E. coli* strains for epidemiological purposes (146).

4.1.5. Toxins

Bacterial toxins are virulence factors produced by a wide variety of pathogens. Toxins help the pathogen spread to deeper tissues after disrupting cellular integrity, accessing nutrients within the host cells, or escaping the immune response (133). The essential toxins studied in the present work are as follows:

4.1.5.1. Cytotoxic necrotising factor 1 (CNF1)

CNF1 is a 115 kDa AB-type toxin that catalyses the deamidation of a conserved glutamine residue in three members of the Rho family of GTP-binding proteins, RhoA, Cdc42, and Rac. Rho GTPases are small proteins involved in several cellular processes, including the regulation of transcription, cell polarity, cell cycle progression, and inflammation. Activation of these regulators leads to cellular changes such as cytoskeleton rearrangements, cell cycle arrest, and disruption of host cell signalling pathways (133,152,153).

CNF1 consists of a cell-binding domain and the C-terminal catalytic domain. Activation and subsequent degradation of Rho GTPases, particularly Rac, promotes the internalisation of bacteria into urothelial cells.

Therefore, CNF is linked with severe UTIs by UPEC and is related to different meningitis-causing *E. coli* strains (133,154).

4.1.5.2. α-Haemolysin (HlyA)

HlyA is a 110 kDa toxin belonging to the class of RTX toxins and is encoded by the *hlyCABD* operon. It forms pores in the cell membrane of the host erythrocytes. Osmotic lysis is triggered by the loss of intracellular K^+ ions and the resulting influx of cations and water (138,153).

However, HlyA also has cytotoxic activity in nucleated cells, including the immune, endothelial, and epithelial cells of the urinary tract. In these cells, HlyA stimulates the activity of serine proteases and caspases, which mediate the degradation of paxillin incducing apoptosis.

In upper UTIs caused by UPEC strains, HlyA induces bladder epithelial cell exfoliation and promotes bacterial release. It also plays a cytotoxic and pro-inflammatory role by causing secretion of the cytokines IL-6 and IL-8 and altering Ca^{2+} influx into the membrane. Combining these two effects, weakens the renal epithelium and favours the passage of bacteria into the blood (133,155).

4.1.5.3. Colibactin

Colibactin is a secondary metabolite synthesised by polyketide synthases and encoded by a 54-kb genomic island designated *pks* (Figure 23). This toxin induces DNA double-strand breaks, chromosomal aberrations, and cell cycle arrest in the G2/M phase (156).

The *pks* genomic island is commonly observed in *E. coli* strains of phylogroup B2, including ExPEC, and has been found in isolates from the intestinal microbiota, septicaemia, neonatal meningitis, and UTIs. The *pks* island is frequently associated with other virulence factors, including other cyclomodulins, adhesins, haemolysins, toxins, and siderophores (156,157).

The frequent detection of the *pks* island in *E. coli* strains from biopsies of patients with colorectal cancer has made it possible to link the presence of colibactin to this type of cancer in humans (155,157,158)

However, the presence of colibactin in species other than *E. coli* is steadily increasing. The first report of the existence of *pks* in *K. pneumoniae* strains was published in 2009 (159). Later, several studies linked the presence of the *pks* locus to gut colonisation and mucosal invasion by *K. pneumoniae* (160). The *pks* genomic island has been reported to be encoded on the chromosomal MGE ICE*Kp*10, one of the 14 integrative and conjugative elements (ICEs), highly abundant in hvKp, linking colibactin to this phenotype (161).

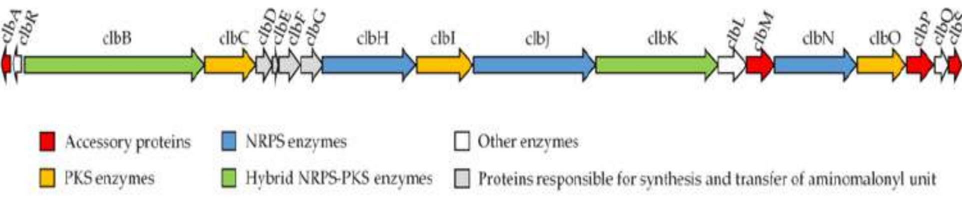


Figure 23. Organisation of the *pks* genomic island. (Adapted from 156: p.4)

4.2. Specific virulence factors of *K. pneumoniae*

4.2.1. Type 3 fimbriae

Type 3 fimbriae, encoded by the *mrk* gene cluster (*mrkABCDF*), are expressed by most clinical *K. pneumoniae* isolates and mediate adhesion and biofilm formation on biotic and abiotic surfaces (127). Most of the structure consists of MrkA subunits, with the adhesion unit MrkD at the top (Figure 24). In contrast to type 1 fimbriae, type 3 fimbriae are "mannose-insensitive" and therefore, do not bind mannose (128). In addition to

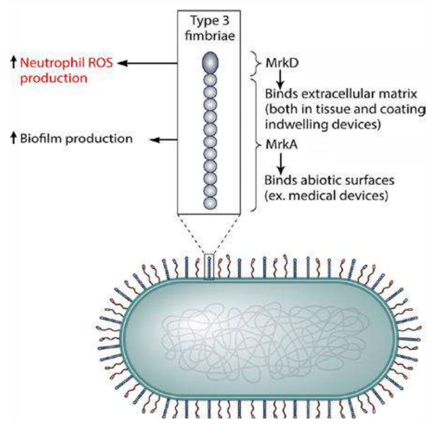


Figure 24. Type 3 fimbriae. (Adapted from 128: p.640)

Klebsiella species, type 3 fimbriae are also common in *Enterobacter, Serratia, Proteus,* and *Providencia* isolates (162).

4.3. Specific virulence factors of *E. coli*

4.3.1. Curli fimbriae

Curli amyloid fibres are composed of numerous CsgA subunits, which after transport across the outer membrane, polymerise with the help of the CsgB subunit to form surface-exposed functional fibres (Figure 25) (133). Curli fimbriae are produced by *E. coli* and *S. enterica* serovar Typhimurium under nutrient limitation, reduced oxygen, and < 30 °C. They play a central role in forming complex colony morphology on agar plates and biofilm formation. In this sense, curli-deficient strains form shallow biofilms compared to the mature biofilms formed by curli-expressing strains. Moreover, curli fimbriae, together with cellulose, make up to 85% of the biomass of the *E. coli* biofilms (133,163). By evading the immune system, curli mediate invasion into host cells and trigger strong immune responses and inflammation by activating the immune Toll-like receptor (TLR) 2 (164,165).

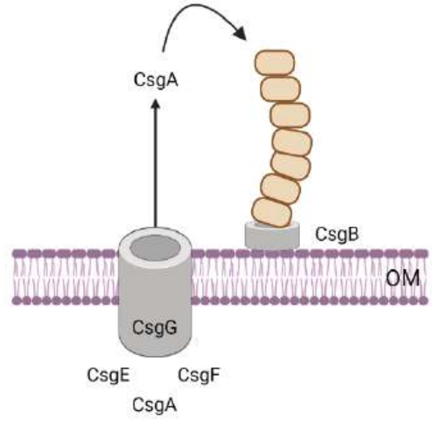


Figure 25. Curli fimbriae.
This figure was created with BioRender.com.

4.3.2. S-fimbriae

Although type 1 or P-fimbriae are prevalent in ExPEC, S-fimbriae are also found in these bacteria (133). S-fimbriae, or sialic acid-specific fimbriae, are encoded by the *sfa* operon (Figure 26), which consists of nine genes and are composed of a major subunit SfaA and three minor subunits SfaG, SfaH, and SfaS (166). S fimbriae bind to α-sialyl-2-3-β-galactose (NeuAc-α 2,3-Gal)-containing receptors on erythrocytes and renal tubular epithelial cells (133).

They have most frequently been found in *E. coli* isolates associated with UTIs, meningitis, and sepsis. However, they can also bind receptors in the brain endothelium, colon, and ileal enterocytes (166).

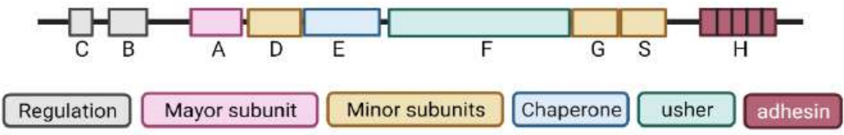


Figure 26. The *sfa* operon. This figure was created with BioRender.com.

4.3.3. P-fimbriae

P-fimbriae are mannose-resistant fimbriae that mediate the attachment of *E. coli* to epithelial urinary cells and erythrocytes. Therefore, P-fimbriae are classically associated with pyelonephritis and bloodstream infections (133).

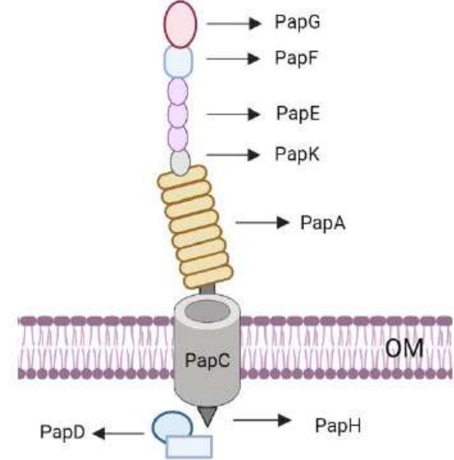


Figure 27. P-fimbriae. This figure was created with BioRender.com.

They are encoded by the *pap* locus and have galabiose (Gal(α1-4)Gal-) specific receptors in the distal PapG unit. The tip is composed of approximately 50 repeat subunits of the PapE protein, and PapA forms the stem. PapF, PapK, and PapH subunits are also present in small amounts (Figure 27) (167,168).

4.3.4. Poly-β-(1,6)-N-acetylglucosamine (PNAG)

The exopolysaccharide PNAG is a non-fimbrial adhesin that plays a crucial role in biofilm formation, pathogenicity, and virulence. It is one of the most important polysaccharides in *E. coli* biofilms (169,170) and mediates cell-

to-cell adhesion and attachment to surfaces (117). Its function in *E. coli* requires the gene products of the *pgaABCD* operon, all of which are necessary for biofilm formation (171). Its biosynthesis is regulated by signalling molecules, such as c-di-GMP, which play a special role in post-translational activation (170).

PNAG is synthesised in the cytoplasm from the nucleotide-sugar precursor UDP-GlcNAc. The polymer is transported across the inner membrane via PgaCD, deacetylated by PgaB in the periplasm, and exported through the PgaA porin (Figure 28) (172).

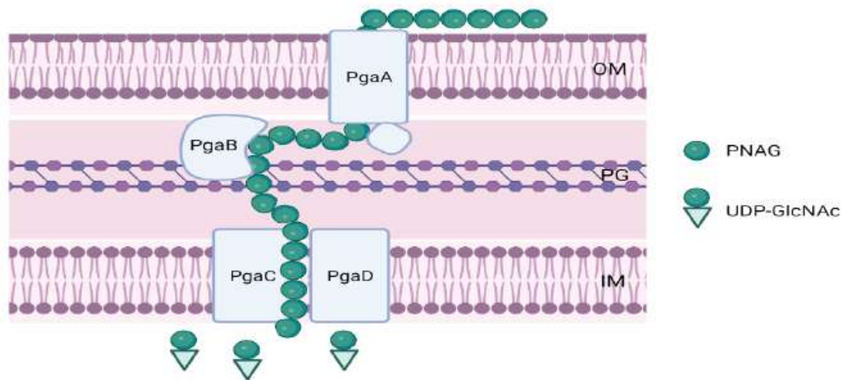


Figure 28. Biosynthesis of PNAG. This figure was created with BioRender.com.

4.3.5. Flagella (H antigen)

The flagellum is a locomotor organelle that enables bacteria to move from nutrient-poor to nutrient-rich environments (14).

A flagellar filament consists of a basal body embedded in the cell wall and two axial structures, the hook and filament, which are joined at the hook–filament junction. The basal body consists of the MS ring, rod, and L- and P-rings (Figure 29). Components of the axial structures are exported from the cell by the flagellar type III secretion system (173,174). Flagella enable movement in a straight line by rotating the filaments counterclockwise (swimming mode), or on the other hand, in tumbling mode, the bacteria rotate their bodies clockwise (175).

Flagellin, also known as H-antigen (FliC protein, encoded by the *fliC* gene in *E. coli*), is a crucial surface antigen for *E. coli* serotyping (14). In *E. coli*, 53 H flagellar antigens have been defined (176).

As mentioned in section 1.2.1, several studies have shown that flagella influence virulence by releasing virulence factors and mediating biofilm formation, bacterial adhesion, and invasion. In addition, flagellin promotes a pro-inflammatory response as it is recognised by TLR-5, which is expressed on the surface of mammalian cells (14).

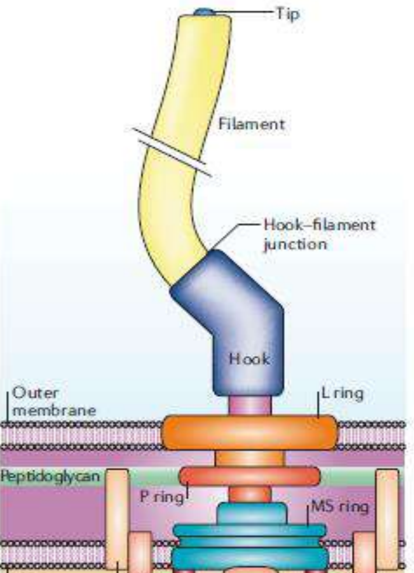


Figure 29. Flagellar structure.
(Adapted from 174: p.785).

5. Antimicrobial therapy and resistance

The discovery of antibiotics was one of the most significant events in human health. For decades, antibiotics have saved many lives and helped control infectious diseases causing significant morbidity and mortality worldwide (177–179).

Selman Waksman first used the term 'antibiotic' in 1941 to refer to small molecules produced by a microbe to counteract the growth of other microorganisms (180). Antibiotics have specific targets, including the bacterial cell wall (e.g., penicillin), cell membrane (e.g., daptomycin), protein synthesis (e.g., gentamicin), DNA and RNA synthesis (e.g., ciprofloxacin), and folic acid metabolism (e.g., trimethoprim) (Figure 30).

However, bacteria use various strategies to evade the attack of antibiotics. Some of these strategies include (181):

(i) Efflux pumps that transport antibiotics from inside to outside the bacterial cell.
(ii) Target modifications that prevent the efficient binding of the antibiotic but allow the target to perform its normal function.
(iii) Inactivating enzymes that inhibit or modify various antibiotics.
(iv) Immunity and bypass, in which antibiotics or their targets are attached to proteins that prevent binding between the antibiotic and its target.

In addition, bacteria can acquire foreign DNA that codes for ARGs and transfer it to others via HGT. Thus, HGT is involved in the spread of resistance determinants and can occur through various mechanisms: transformation (with free DNA), transduction (by bacteriophages), or conjugation (involving plasmids) (Figure 31) (182,183).

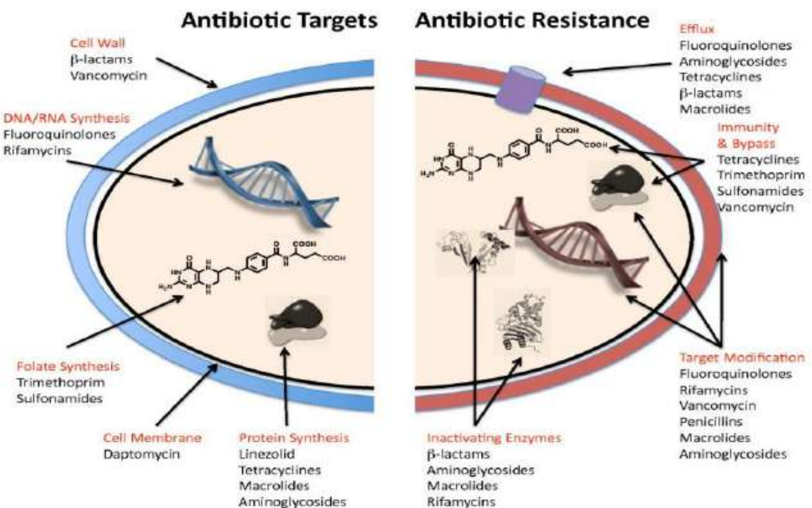


Figure 30. Antibiotic targets and antibiotic resistance mechanisms. (Adapted from 181: p.2).

In **transformation** (Figure 31a), free DNA from a bacterial donor is incorporated into a recipient bacterium, which can integrate this DNA into its chromosome or a plasmid (183,184). Transformation has the disadvantage that naked DNA can be rapidly degraded, shortening the time for successful transformation (185). The process involves type II and type IV (T2SS and T4SS) secretion systems and type IV pili, by which the bacteria take up the DNA from the surface into the cytoplasm (186).

Transduction (Figure 31b) is the HGT mechanism mediated by bacteriophages (183). The bacteriophage injects the DNA content into another bacterium for further replication (186). In this way, ARGs can be transferred and later integrated into the recipient cell's chromosome (184).

Conjugation (Figure 31c) occurs through momentary and direct contact between two bacterial cells. The cells form a conjugation bridge, whereby the genetic material is transferred from the donor to the recipient. Plasmids and transposons are the most common MGEs used in conjugation (183–185). **Plasmids** can replicate independently and harbour a collection of ARGs and VFGs (185). **Transposons** are DNA sequences that can jump from

one place to another, from chromosome to plasmid and vice versa, allowing the transfer and permanent addition of genes, including those encoding for antibiotic resistance (184). Furthermore, some transposons contain **integrons** which are genetic elements that can capture, accumulate, and express ARGs. Integrons provide an efficient mechanism to insert new genes into bacterial chromosomes, ensuring their expression (183).

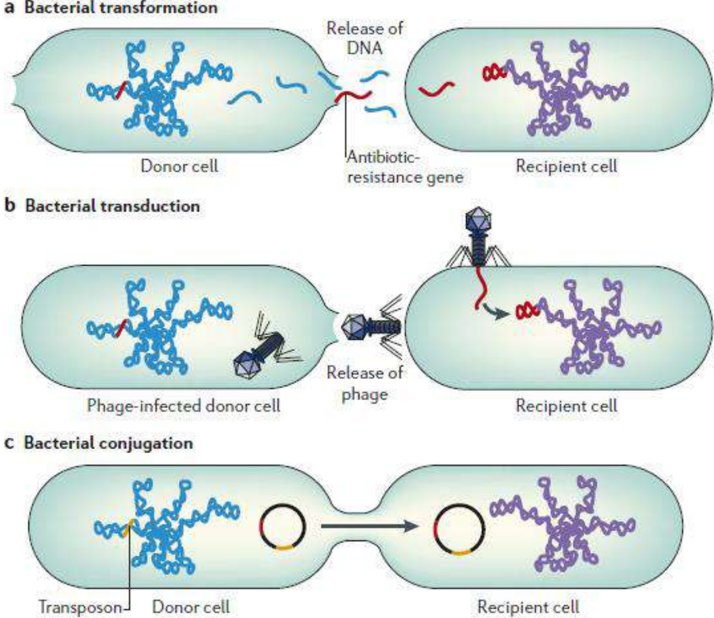


Figure 31. Horizontal gene transfer mechanisms. A. Bacterial transformation. **B.** Bacterial transduction. **C.** Bacterial conjugation. (Adapted from 184: p.38).

Some of the antimicrobial agents most commonly used to treat infections caused by *E. coli* and *K. pneumoniae*, and the corresponding resistance mechanisms are briefly explained in the following sections.

5.1. Aminoglycosides

Aminoglycosides belong to the group of antibiotics that inhibit protein synthesis by binding irreversibly and with high affinity to the A-site of the small 30S ribosomal subunit (187). This antibiotic family includes streptomycin, kanamycin, tobramycin, gentamicin, among others, and is

used in a wide range of bacterial infections. Aminoglycosides are active against most aerobic Gram-negative bacteria but lack significant activity against *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Pasturella multocida* and anaerobic organisms. Gram-positive bacteria are relatively resistant to aminoglycosides, and thus, their use against these microorganisms is limited (188).

Although they are not recommended as first-line agents due to their nephrotoxicity, aminoglycosides are used in UTIs and as empirical therapy in severe sepsis or in the treatment of MDR pathogens for which there is no suitable alternative (188). Moreover, inhaled aminoglycosides have been used in severe VAP cases (189).

Bacteria have developed mechanisms of resistance to aminoglycosides, including:

(i) Enzymatic drug modification by aminoglycoside-modifying enzymes such as acetyltransferases, phosphotransferases and nucleotidyltransferases. Among these, aminoglycoside N-acetyltransferases (AACs) are clinically meaningful and are divided into four main groups based on the position of acetylation: AAC(1), AAC(2'), AAC(3), and AAC(6') (187,190,191).

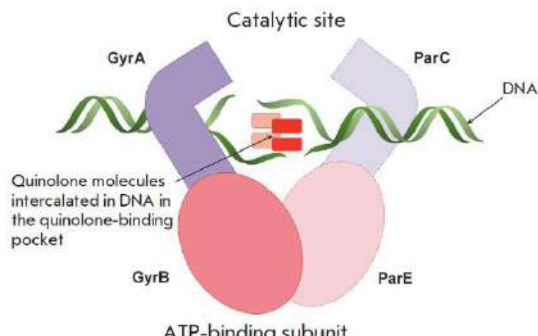
(ii) Mutation and modification of the ribosomal target by 16S rRNA methyltransferases modify rRNA residues that block the binding of the antibiotic to the target (188).

(iii) Several members of the RND family of efflux pumps have been associated with intrinsic aminoglycoside resistance (188).

5.2. Quinolones

Quinolones are the most effective synthetic antimicrobial agents with broad-spectrum activity that inhibit bacterial topoisomerases. They are used to treat infections caused by both Gram-positive and Gram-negative bacteria. Fluoroquinolones such as ciprofloxacin, levofloxacin, and norfloxacin are the agents most commonly used against *Enterobacterales* (192).

Quinolones act on bacterial DNA gyrase and topoisomerase IV, which are crucial for replication, transcription, recombination, and the remodelling of condensed DNA (Figure 32). Therefore, quinolones convert these enzymes into toxic enzymes that fragment the bacterial chromosome. Consequently, quinolones are associated with the inhibition of DNA and RNA synthesis (193).



F **Figure 32. Mechanisms of quinolone action** (Adapted from 193: p.36).

The mechanisms used by bacteria to evade quinolones include:

(i) Overexpression of efflux pumps that reduces cellular concentrations of quinolones (194).

(ii) Under-expression of porins reduces the ability to take up the drug, reducing the antibiotic concentration in the bacterial cell (192).

(iii) Plasmid-mediated resistance leads to the synthesis of proteins that interfere with quinolone–enzyme interactions, alter drug metabolism, or increase quinolone efflux. Three families of proteins are associated with plasmid-mediated quinolone resistance: the Qnr family with more than 100 Qnr variants; the plasmid-encoded protein associated with quinolone resistance *aac(6′)-Ib-cr*; and the efflux pumps OqxAB, QepA1, and QepA2 (194).

(iv) Mutations in the quinolone resistance-determining region that are located between amino acids 67 and 106 in GyrA or between

amino acids 63 and 102 in ParC. These mutations are also found in GyrB between amino acids 426 and 447 and in ParE between amino acids 420 and 441 (192).

5.3. Trimethoprim-sulfamethoxazole

Trimethoprim is a synthetic antimicrobial agent usually used with sulfamethoxazole that has a synergistic effect and covers a broad antimicrobial spectrum. Both drugs affect bacterial folic acid synthesis by inhibiting two of the key enzymes in this pathway. Thus, sulfamethoxazole inhibits dihydropteroate synthetase, which catalyses the formation of dihydrofolate from para-aminobenzoic acid, while trimethoprim has an inhibitory effect on dihydrofolate reductase, which catalyses the formation of tetrahydrofolate from dihydrofolate (195–197)

The mechanisms of bacterial resistance to one or both molecules include the following (196):

(i) Permeability barrier and/or efflux pumps.
(ii) Inherent insensitive target enzymes.
(iii) Regulatory changes, mutations, or recombination in target enzymes.
(iv) Transferrable resistance by sulfonamide resistance enzymes encoded by *sul1* and *sul2* genes.

5.4. Chloramphenicol

Chloramphenicol is a bacteriostatic antibiotic produced naturally by *Streptomyces venezuelae* and used for infections caused by Gram-positive and Gram-negative bacteria. It inhibits protein biosynthesis by interacting with the bacterial 50S subunit of the ribosome and blocking the binding of amino acyl-tRNA at the A-site (190,198,199).

The mechanisms of resistance to chloramphenicol include:

(i) Production of modifying or inactivating enzymes by acetylation mainly via acetyltransferases or chloramphenicol phosphotransferases.
(ii) Decreased outer membrane permeability.
(iii) Efflux pumps that often act as multidrug extrusion transporters.
(iv) Mutations or modifications of the target sites.

5.5. Fosfomycin

Fosfomycin is a bactericidal antibiotic produced by *Streptomyces* spp. It is effective against both Gram-positive and Gram-negative pathogens. Fosfomycin affects peptidoglycan biosynthesis. Once inside the cell, fosfomycin inhibits the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), which is responsible for catalysing the formation of N-acetylmuramic acid (the precursor of peptidoglycan) by binding N-acetylglucosamine and phosphoenolpyruvate (190,200). Fosfomycin has been used to treat infections caused by MDR bacteria (201).

Fosfomycin resistance is mainly produced by:

(i) Mutations in the *murA* gene.
(ii) Mutations in the uptake systems.
(iii) Acquisition of plasmid-encoded genes that inhibit the antibiotic, being the plasmid-mediated Fos enzymes, essentially FosA and FosB, the most important.

5.6. Colistin

Colistin, also known as polymyxin E, belongs to the antibiotic family of polymixins, a group of cyclic antimicrobial oligopeptides with hydrophilic and lipophilic properties, which comprise five compounds (A, B, C, D, and E). Polymyxin B and polymyxin E are currently used in human and veterinary medicin*E. coli*stin was discovered in Japan in 1949 and is produced by *Bacillus polymyxa*. It is effective against Gram-negative bacteria but is ineffective against Gram-positive bacteria, anaerobic bacteria, and mycoplasmas. It is considered the antimicrobial agent of last choice for

infections caused by MDR bacteria. Colistin binds to the phosphate groups of lipid A present in the LPS of Gram-negative bacteria through electrostatic interactions, destroying the integrity of the outer and inner membranes of the bacteria (202–204).

The mechanisms involved in resistance to colistin include:

(i) Modifications of LPS by the addition of cationic groups.
(ii) Mutations in genes associated with the lipid A biosynthesis pathway.
(iii) Porin mutations and overexpression of efflux pumps.
(iv) Overproduction of CPS.
(v) Colistin-inactivating enzymes. The production of colistin-inactivating enzymes has become very important in recent years. The plasmid-mediated colistin resistance *mcr-1* gene was described in *E. coli* in China in 2015 (205). To date, additional *mcr* genes (*mcr-2* to *mcr-9*) have been identified in other *Enterobacterales* (206).

5.7. β-Lactams

β-Lactams are the most commonly used antibiotics in medicine to treat Gram-positive and Gram-negative bacterial infections. The β-lactam ring interacts with the nucleophilic serine of penicillin-binding proteins (PBPs), leading to irreversible acylation of the PBPs, which prevents the formation of transpeptide peptidoglycan linkages (190,207,208).

There are five main classes of antimicrobial β-lactams: penicillins, cephamycins, cephalosporins, carbapenems, and monobactams.

Resistance to β-lactams is associated with:

(i) Modifications of PBPs, the primary mechanism of resistance in Gram-positive bacteria.
(ii) Efflux pumps.
(iii) Decreased cell permeability.

(iv) β-Lactamase enzymes, which are highly important in antimicrobial resistance (AMR), are explained in more detail in this section.

β-Lactamases are enzymes that hydrolyse the β-lactam ring and their genes are encoded on MGEs that are transferable between species. Major β-lactamase families include plasmid-mediated extended-spectrum β-lactamases (ESBLs), AmpC cephalosporinases, and carbapenemases (207,208).

According to the Ambler classification based on their sequence identities, β-lactamases are divided into four groups (A, B, C, D). Classes A, C, and D contain serine in the active site, and class B represents zinc metallo-β-lactamases (MBLs) (207,209).

Class A enzymes are the most common β-lactamases worldwide. Class A enzymes mainly include TEM, SHV, CTX-M, and KPC (207). Mutations in TEM and SHV have produced new variants that enable them to hydrolyse cefotaxime and ceftazidime, giving rise to the ESBL enzymes (210). The CTX-M group is one of the epidemiologically most important ESBL groups and is widespread throughout the world. The CTX-M-type enzymes or cefotaximases are divided phylogenetically into five different clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25). They hydrolyse cefotaxime and ceftriaxone better than ceftazidime, although the spectrum varies depending on the enzyme. In addition, all β-lactamase inhibitors, including avibactam and vaborbactam, can inhibit them (211).

CTX-M ESBLs are commonly found in *E. coli* and *K. pneumoniae* but are also found in other species of *Enterobacterales* and several species of non-fermenters bacteria. CTX-M-15 belongs to the CTX-M-1 group and is the most commonly ESBL found worldwide (209,211). The KPC group has become one of the most frequently detected carbapenemases worldwide. They are not inhibited by clavulanate or tazobactam but are efficiently inhibited by avibactam, relebactam, and vaborbactam. However, it has been described

that mutant resistant strains are resistant to ceftazidime-avibactam via increased catalytic activity for ceftazidime (211).

Class B enzymes, or MBLs, have a broad substrate spectrum that includes all β-lactams except monobactams (aztreonam). Moreover, they are not inhibited by most β-lactamase inhibitors, which is the major challenge for the development of new β-lactamase inhibitors. Metal chelators such as ethylenediaminetetraacetic acid (EDTA) inhibit MBLs. The MBL group includes NDM and VIM as the most important representatives (207,212).

Class C enzymes have an excellent affinity for cephalosporins. Most class C enzymes are not inhibited by classical inhibitors, such as clavulanate, sulbactam, and tazobactam, but are inhibited by the newer avibactam, relebactam, and vaborbactam. Some enzymes belonging to this group are CMY, ADC, and the clinically relevant Amp-C (207,208,211).

Class D enzymes preferentially hydrolyse oxacillin, penicillins, and broad-spectrum carbapenems. They are hardly inhibited by β-lactamase inhibitors, except for avibactam. The most important representatives of this group are the OXA-23 and OXA-48 enzymes, which are responsible for the carbapenem resistance of *A. baumannii* and *Enterobacterales*, respectively (207,211).

On the other hand, according to the Bush-Jacoby-Medeiros classification, β-lactamases are classified according to their functional similarities and substrate and inhibitor profiles. In this system, there are three main groups and several subgroups. The main groups generally correlate with the Ambler classification. Group 1 (Class C) cephalosporinases; group 2 (Classes A and D) broad-spectrum, inhibitor-resistant, ESBLs and serine carbapenemases; and group 3 MBLs (211,213). The nomenclature of clinically relevant β-lactamases is summarised in Table 2.

Table 2. Nomenclature of clinically important β-lactamase enzymes (Adapted from 211: p.5).

Molecular class or subclass	Functional group or subgroup	Common name	Clinically relevant enzymes	Characteristic substrate profile	Characteristic inhibitor profile
A	2a	Penicillinase	PC1/blaZ	Narrow-spectrum PENs	CA, TZB
A	2b	Penicillinase	TEM-1, SHV-1	Narrow-spectrum PENs, early CEPHs	CA, TZB
A	2be	ESBL	TEM-10, SHV-2, CTX-M-15	Narrow-spectrum PENs, early CEPHs, ES-CEPHs, monobactams	CA, TZB, AVI
A	2br	IRT	TEM-30 (IRT-2)	PENs, early CEPHs	TZB, AVI
A	2e	ESBL cephalosporinase	CepA	ES-CEPHs	CA but no ATM
A	2f	Carbapenemase	KPC	All FDA-approved β-lactams	AVI, REL, VAB
A	2f	Carbapenemase	SME	PENs, early CEPHs, carbapenems, monobactams, not ES-CEPHS	CA, AVI, VAB
B1,B3	3a	MBL, carbapenemase	IMP, NDM, VIM, SPM	All PENs, CEPHs, carbapenems, not monobactams	EDTA
B2	3b	MBL, carbapenemase	L1, CphA	Carbapenems preferred	EDTA
C	1	Cephalosporinase	AmpC	CEPHs	ATM, AVI, VAB
D	2d	Oxacillinase	OXA-1	PENs, especially oxacillin/cloxacillin	Variable
D	2df	Carbapenemase	OXA-23, OXA-48, OXA-181, OXA-232	PENs, especially oxacillin/cloxacillin, carbapenems	AVI (OXA-48)

ESBL: extended-spectrum β-lactamase; IRT: inhibitor-resistant TEM; MBL: metallo-β-lactamase; CEPH: cephalosporin; ES-CEPHS: expanded-spectrum cephalosporins; PEN: penicillin; FDA: Food and Drug Administration; EDTA: Ethylenediaminetetraacetic acid; ATM: aztreonam; AVI: avibactam; CA: clavulanic acid; REL: relebactam; TZB: tazobactam; VAB: vaborbactam.

5.8. β-Lactamase inhibitors

Due to the emergence of β-lactamases, it was necessary to search for molecules that could inhibit these enzymes. For this reason, the discovery of molecules called β-lactamase inhibitors and their combination with some β-lactams was the main strategy to achieve this.

Clavulanic acid was the first inhibitor identified against most class A enzymes found in *Enterobacterales*. After discovering clavulanic acid, **sulbactam** and **tazobactam** were successfully approved in combination with various β-lactams (214). Thus, **amoxicillin-clavulanate**, **ampicillin-sulbactam**, and **piperacillin-tazobactam** have been widely used for community and healthcare-associated infections caused by β-lactamase-producing bacteria. However, their limited spectrum of activity has driven the development of new inhibitors. Among them, avibactam was the first to be used with ceftazidime. **Avibactam** is a potent inhibitor of class A β-lactamases including KPC, and class C enzymes. Then, the development of cyclic boronates with the ability to inhibit serine β-lactamases introduced the use of meropenem in combination with the cyclic boronate vaborbactam. **Vaborbactam** inhibits class A β-lactamases but not OXA or MBL enzymes (207,208).

Two diazabicyclooctane molecules have been discovered in recent years, **relebactam** and **nacubactam**. Relebactam has been approved in combination with imipenem as an inhibitor of class A, C, and certain D β-lactamases, although it is not active against OXA-48. The new combination meropenem/nacubactam is currently being developed by NacuGen Therapeutics and is in the early clinical phase (215,216).

6. Relationship between virulence and antimicrobial resistance

It was assumed that increasing antimicrobial resistance was significantly associated with lower virulence and fitness. However, several studies have shown the opposite: increased resistance can be beneficial rather than detrimental to bacteria (217).

In the first scenario, the studies conducted by Horcajada *et al.* and Vila *et al.* revealed that some quinolone resistant UPEC strains had fewer VFGs and a lower capacity to invade the urinary tract than quinolone-susceptible strains (218,219). Similarly, the study by Rudkin *et al.* revealed that expression of the *mecA* gene, which is responsible for methicillin resistance in *S. aureus*, impairs the *agr* QS system and reduces the ability of MRSA to secrete toxins. Thus, the *mecA* gene acts not only as a resistance gene but also as a virulence regulator (220). In another study, the expression of OXA-24, OXA-10-like, and SFO-1 β-lactamases in *E. coli* was found to induce several changes in peptidoglycan composition, leading to a significant fitness cost (221).

On the other hand, the theory on how antimicrobial resistance increases virulence is supported by several facts. First, MGEs can transfer ARGs and VFGs (222). In addition, some ARGs are often embedded in transposons that can mobilise these genes into the chromosome, reducing fitness costs and enabling the acquisition of new plasmids that may harbour virulence genes (223). Second, bacteria can evolve and adapt to reduce the cost of fitness, either through compensatory mutations that restore virulence without loss of antibiotic resistance or by regulating the expression of resistance (224). In addition, some of these mutations that lead to antimicrobial resistance may not incur fitness costs (223). Third, the regulation of virulence and antimicrobial resistance is complex, as the regulation of virulence genes can often influence the expression of resistance genes and vice versa. Transcription factors, post-transcriptional modifications, stress responses, quorum sensing, etc., mediate such regulation (217).

Some examples supporting this include the successful case of the CTX-M-producing *E. coli* ST131 clone, which has a competitive advantage over other less virulent or more susceptible isolates of *E. coli* and has been found worldwide (223,224). Similarly, studies with *S. enterica* serovar Typhimurium have shown that avirulent isolates resistant to nalidixic acid, streptomycin, or rifampicin developed compensatory mutations at a second site that restored virulence (225). Miskinyte *et al.* reported that *E. coli* strains resistant to streptomycin due to a single point mutation could survive better inside macrophages, suggesting that antibiotic-resistant bacteria can evade the host immune system (226). Guillard *et al.* concluded that *E. coli* strains that acquired the QnrA3-producing plasmid, which belongs to the plasmid-mediated quinolone resistance (PMQR) group, not only showed increased fitness but also integrated *qnrA3* into the chromosome, resulting in higher resistance to quinolones (223).

In either scenario, new strategies to control the spread of AMR must also focus on controlling the virulence spread.

VII. Work justification, Hypotheses and Objectives

Biofilms are a major global health problem due to their inherent tolerance to antimicrobial treatments (227). Although some biofilms are monomicrobial, they usually form polymicrobial communities, including human infections. Moreover, the underlying mechanisms of biofilm formation are not fully understood, and many virulence genes or even antimicrobial resistance genes may be involved in this process. Identifying new genes that could be associated with biofilm formation and the discovery of novel molecules to inhibit or eradicate biofilms is currently a priority research goal.

For this reason, this dissertation focuses on approaching the study of biofilm formation from different angles. First, we analysed the interactions between species in UTIs, specifically the biofilm formed by *K. pneumoniae* and *E. faecalis*. Second, we searched for new genes involved in biofilm formation in *E. coli* using random transposon insertion. We also investigated a new molecule of natural origin that could be considered in the future as a means of treating biofilms formed not only by *E. coli* but also by other Gram-negative and Gram-positive bacteria. Finally, we analysed whether the origin of the strains, the antimicrobial resistance profiles, or the presence or absence of different virulence determinants in a collection of *E. coli* and *K. pneumoniae* strains could be related to their ability to form biofilms. Thus, four hypotheses and objectives are proposed in this thesis:

Hypothesis 1

Antagonistic interactions modulate the polymicrobial biofilm formed by *K. pneumoniae* and *E. faecalis*.

Objective 1

To analyse the inter-species interactions within the polymicrobial biofilm formed by *K. pneumoniae* and *E. faecalis* **(Paper 1).**

Hypothesis 2

The random transposon insertion method could facilitate the discovery of new genes involved in *E. coli* biofilm formation that could be considered new therapeutic targets.

Objective 2

To identify new genes involved in biofilm formation in *E. coli* by transcriptomic and proteomic analysis of isogenic mutants generated by transposition (**Paper 2**).

Hypothesis 3

New molecules of natural origin could have bactericidal and antibiofilm effects, helping combat antibiotic resistance and biofilm formation.

Objective 3

To evaluate the antibacterial and antibiofilm activity of a fruit extract containing proteolytic enzymes against various Gram-positive and Gram-negative bacteria (**Manuscript 1**).

Hypothesis 4

The origin of the bacterial strains, their antimicrobial resistance profiles, and the presence or absence of different virulence genes could be related to biofilm formation.

Objective 4

To characterise a collection of clinical strains of *E. coli* and *K. pneumoniae* for antimicrobial resistance and virulence determinants to elucidate the relationship between these characteristics and the ability to form biofilms (**Paper 3 and Paper 4**).

VIII. Results

Paper 1. *Enterococcus faecalis* inhibits *Klebsiella pneumoniae* growth in polymicrobial biofilms in a glucose-enriched medium

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Authors: Victoria Ballén, Carlos Ratia, Virginio Cepas & Sara M. Soto

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Hypothesis: Antagonistic interactions modulate the polymicrobial biofilm formed by *K. pneumoniae* and *E. faecalis.*

Objectives: To analyse the inter-species interactions within the polymicrobial biofilm formed by *K. pneumoniae* and *E. faecalis*.

Materials and methods: Different bacterial strains isolated from urinary tract infections were used in this study. The strains were previously identified by MALDI-TOF mass spectrometry, their antimicrobial profile was tested according to the M100 guidelines (CLSI. 2019), and their ability to form biofilms was characterised according to the protocol by Stepanovic *et al.* (2007). Biofilm tests were performed, including surface adhesion, quantification of mono- and polymicrobial biofilms, calculation of the percentage of inhibition, quantification of viable cells, and calculation of the competitive index. We performed minimum inhibitory concentrations and antibiofilm assays to evaluate the effect of lyophilised cell-free supernatant of *E. faecalis* on planktonic cells and mature *K. pneumoniae* biofilms. Finally, competition in planktonic cultures, polymicrobial

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biofilms using *E. faecalis* mutant strains defective in lactic acid, and analysis of interspecies interactions using pooled human urine were also investigated.

Results: Although *K. pneumoniae* was the most adherent strain, it could not maintain dominance in the polymicrobial biofilm due to the lactic acid produced by *E. faecalis* in a glucose-enriched medium. This result was supported by the use of the *E. faecalis* V583 *ldh-1*/*ldh-2* double mutant (which does not produce lactic acid), which did not inhibit the growth of *K. pneumoniae*. Lyophilised cell-free supernatants derived from *E. faecalis* biofilms showed antimicrobial/antibiofilm activity against *K. pneumoniae*. Conversely, there were no significant differences in planktonic polymicrobial cultures.

Conclusions: *K. pneumoniae* and *E. faecalis* were found to compete when cultivated in biofilms in a high glucose environment. Both microorganisms produced more biomass in monomicrobial biofilms than in polymicrobial biofilms. *E. faecalis* showed inhibitory activity against *K. pneumoniae* altering the pH of the medium due to the production of lactic acid, which has detrimental effects on *K. pneumoniae* without affecting its growth.

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Enterococcus faecalis inhibits *Klebsiella pneumoniae* growth in polymicrobial biofilms in a glucose-enriched medium

Victoria Ballén, Carlos Ratia, Virginio Cepas and Sara M. Soto

ISGlobal, Barcelona Center for International Health Research (CRESIB), Hospital Clinic - Universitat de Barcelona, Barcelona, Spain

ABSTRACT

Catheter-related urinary tract infections are one of the most common biofilm-associated diseases. Within biofilms, bacteria cooperate, compete, or have neutral interactions. This study aimed to investigate the interactions in polymicrobial biofilms of *Klebsiella pneumoniae* and *Enterococcus faecalis*, two of the most common uropathogens. Although *K. pneumoniae* was the most adherent strain, it could not maintain dominance in the polymicrobial biofilm due to the lactic acid produced by *E. faecalis* in a glucose-enriched medium. This result was supported by the use of *E. faecalis* V583 *ldh-1/ldh-2* double mutant (non-producer of lactic acid), which did not inhibit the growth of *K. pneumoniae*. Lyophilized cell-free supernatants obtained from *E. faecalis* biofilms also showed antimicrobial/anti-biofilm activity against *K. pneumoniae*. Conversely, there were no significant differences in planktonic polymicrobial cultures. In summary, *E. faecalis* modifies the pH by lactic acid production in polymicrobial biofilms, which impairs the growth of *K. pneumoniae*.

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KEYWORDS
Polymicrobial biofilms; interspecies interactions; competition; lyophilized cell-free supernatant; lactic acid

Introduction

Biofilms are microbial communities of surface-attached cells embedded in a self-made matrix of extracellular polymeric substances that play an important role in a variety of infections, including catheter-related infections (Dybowska-Sarapuk et al. 2017). In this context, although indwelling device-related urinary tract infections are one of the most common biofilm infections of the urinary system (Kirmusaoglu et al. 2017), it may not lead to a high mortality rate. Nevertheless, they represent a challenge for the health care system as they increase morbidity and treatment costs (Frank et al. 2009).

The microorganisms that usually colonise indwelling urinary catheters and develop biofilms are *Staphylococcus epidermidis, E. faecalis, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa* and *K. pneumoniae* (Sabir et al. 2017). Most research studies have focused on monomicrobial biofilms to understand the mechanisms involved in biofilm development (Lee et al. 2014). However, the majority of biofilms appear to be a diverse community of microorganisms (Elias and Banin 2012), and the clinical field is no exception. A large number of biofilms involved in catheter-associated urinary tract infections (CAUTI) are formed by polymicrobial communities (Azevedo et al. 2017). Galván et al. (2016) also reported that some of the dual-species associations with higher prevalence in urine samples are *K. pneumoniae/E. coli, E. coli/E. faecalis, K. pneumoniae/E. faecalis*, and *K. pneumoniae/P. mirabilis* - accounting for 26%, 10%, 8.5%, and 7% of cases, respectively (Galván et al. 2016).

Within biofilms, social interactions of cooperation or competition between cells occur and could cause many changes in community behaviour (Flemming et al. 2016). In fact, in contrast to cultures of planktonic cells, these interactions are allowed by high cell concentrations and the diffusion limitations present in biofilms (Rendueles and Ghigo 2012). New technological developments have made it possible to study the diversity of highly complex microbial communities. Nevertheless, there is a lack of knowledge about the impact of interspecies relationships on the infection process (Røder et al. 2016).

The present study focused on the evaluation of interspecies interactions in polymicrobial biofilms of *K. pneumoniae* and *E. faecalis*. *K. pneumoniae* is a Gram-negative, encapsulated, non-motile, facultative anaerobe, and rod-shaped bacterium (Guentzel 1996).

CONTACT Sara M. Soto sara.soto@isglobal.org

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Table 1. Bacterial strains used in this study.

	Strain	Resistance antimicrobial profile[a]	Biofilm formation	OD 580nm[b]
K. pneumoniae	ATCC 13883	Susceptible	Weak	0.31 ± 0.02
	Kp AT	AN, ATM, CAZ, CIP, CTX, FEP, MEM, SXT, PTZ	Strong	1.48 ± 0.13
	Kp 188	Susceptible	Weak	0.31 ± 0.05
	Kp 529	AMC, ATM, CAZ, CIP, CTX, FEP, GM, SXT	Weak	0.30 ± 0.08
	Kp 725	AMC, ATM, CAZ, CIP, CTX, FEP, SXT	Moderate	0.58 ± 0.02
E. faecalis	Ef 2	Susceptible	Strong	1.38 ± 0.18
	Ef 3	Susceptible	Strong	1.49 ± 0.18
	Ef 5	Susceptible	Strong	1.40 ± 0.09
	V583	VAN	Strong	1.04 ± 0.09
	V583 Δ*ldh1*	VAN	Strong	0.72 ± 0.08
	V583 Δ*ldh2*	VAN	Strong	0.97 ± 0.07
	V583 Δ*ldh1*/Δ*ldh2*	VAN	Strong	0.69 ± 0.09

[a]AMC: Amoxicillin/clavulanic acid, AN: Amikacin, ATM: Aztreonam, CAZ: Ceftazidime, CIP: Ciprofloxacin, CTX: Cefotaxime, FEP: Cefepime, GM: Gentamicin, MEM: Meropenem, PTZ: Piperacillin-tazobactam. SXT: Trimethoprim-sulfamethoxazole. VAN: Vancomycin.
[b]Biofilm in LB broth supplemented with 1% glucose, measured with CV staining after incubation for 48 h and expressed as means ± SDs.

Different virulence factors are associated with the biofilm formation of *K. pneumoniae*, the most important of which are capsular polysaccharides or type 1- and type 3-fimbriae (Bei et al. 2014). On the other hand, *E. faecalis* is a Gram-positive, non-motile, facultative anaerobe, and round-shape bacterium. Previous research reported some virulence factors associated with biofilm formation, including *esp*, *gelE*, and *asa1* genes, which favour cell adhesion, surface colonisation, aggregation and persistence in the urinary tract (Paganelli et al. 2012). These two pathogens are among the most prevalent in urinary tract infections, but information on their specific interaction in biofilms is still scarce. In addition, *K. pneumoniae* is one of the species listed as "priority pathogens" by the World Health Organization to help prioritise the research and development of new and effective antibiotics (World Health Organization 2017).

Materials and methods

Bacterial strains

The strains used in this study are listed in Table 1. The clinical strains were isolated from midstream urine samples and belong to a collection of the authors' research group. The main results of this study focus on one *K. pneumoniae* (AT) and four *E. faecalis* (2, 3, 5, and V583), strins as they are strong biofilm-forming strains. The results of the other *K. pneumoniae* strains analysed (Kp ATCC 13883, Kp 188, Kp 529, Kp 725) are presented as Supplementary material.

E. faecalis V583 wild type (wt) and its mutants with a deletion in *ldh-1* (Δ*ldh1*), *ldh-2* (Δ*ldh2*), or both genes (Δ*ldh1*/Δ*ldh2*) were used to complete this study, on the influence of lactic acid production on the polymicrobial biofilms.

All strains were previously identified by MALDI-TOF mass spectrometry, their antimicrobial profile was tested according to the M100 guidelines (CLSI. 2019) and their biofilm-forming ability was characterised according to the protocol of Stepanović et al. (2007) where a cut-off value (ODc) was established as three standard deviations (SDs) above the mean OD value of the negative control. The interpretation was as follows: optical density of the strain (OD) $\leq$ ODc, the strain was not considered to be a biofilm producer; ODc < OD $\leq$ 2 × ODc, was considered a weak biofilm producer; 2 × ODc < OD $\leq$ 4 × ODc, was considered a moderate biofilm producer; 4 × ODc < OD, was considered a strong biofilm producer. Biofilm formation was measured in three technical and biological replicates.

Biofilm assays

Adhesion to abiotic surfaces

Adhesion to polystyrene plates was performed according to the protocol described by DiMartino et al. (2003) with some modifications. Briefly, flat-bottomed non-treated 6-well microtiter plates (VWR International) were filled with two ml of a suspension of 10^8 colony forming units (CFUs) per ml prepared in 1 × phosphate-buffered saline (PBS) (pH 7.2) and incubated for 30 min, and 1, 2 and 3 h at 37 °C. After incubation, each well was washed with 1 × PBS. Adherent bacteria were released by sonication for 30 s in an ultrasonic bath (Branson 3510, Marshall Scientific) and quantified using 10-fold serial dilutions and conventional plating on Luria Bertani agar (Miller's LB AGAR, Condalab) for *K. pneumoniae* and BD Columbia agar with 5% Sheep Blood (Becton Dickinson) for *E. faecalis*. The plates were then

incubated at 37 °C for 18-24 h. Bacterial adhesion was expressed as a percentage of the original inoculum adhering to the well. The strains were considered strongly adherent to a surface if the percentage of adherent bacteria was >1% compared with the original inoculum. The experiment was performed in three technical and biological replicates.

Development and quantification of Mono- and polymicrobial biofilms

The development of the mono- and polymicrobial biofilms was performed with a modified protocol previously described by Makovcova et al. (2017). Briefly, the bacterial strains were cultured in 10 ml of trypticase soy broth (TSB, Condalab) overnight at 37 °C with shaking at 180 rpm. The bacterial cells were then pelleted at 4,000 ×g for 20 min, and the pellet was resuspended in 5 ml of fresh TSB supplemented with 1% glucose. The optical density (OD_{600nm}) of the bacterial suspensions was measured with the Ultrospec 10 cell density meter (Amersham Biosciences) and adjusted to a final concentration of $\sim 1 \times 10^7$ CFU ml^{-1}. An equal volume of each strain was combined to achieve the polymicrobial culture ratio 1:1. The biofilms were cultured in flat-bottomed non-treated 6-well microtiter plates (VWR International). A sterility control (culture medium without inoculum) was provided. All plates were covered with adhesive lids to avoid evaporation and then incubated under static conditions at 37 °C for 30 min, and 1, 2, 3, 4, 8, 24, 48, and 72 h. The medium was replaced every 24 h by fresh, supplemented TSB broth. The initial inoculum of each strain was confirmed by colony counts.

After incubation, biofilm production was quantified following a previously described protocol (Cepas et al. 2019). Briefly, the remaining culture was carefully removed and each well was gently rinsed with 1 × PBS. Then, all the plates were dried at 65 °C to fix the biofilm to the surface. The biofilms were stained with crystal violet (CV) (2% v/v) and incubated for 10 min at room temperature. Afterwards, the CV was removed, and the plates rinsed once with 1 × PBS and dried at 65 °C for 60 min. Biofilm formation was quantified by eluting the CV fixed to the biofilm in 33% glacial acetic acid and the absorbance of each well was measured at 580 nm (OD_{580nm}) with a microplate spectrophotometer (EPOCH 2 microplate reader; BioTek, VT). The experiment was carried out in three technical and biological replicates.

Percentage inhibition in biofilm formation

Following the protocol described by Reece et al. (2018), the percentage inhibition in biofilm formation was determined as the difference between the OD_{580nm} of each mixed biofilm and the sum of the OD_{580nm} values of biofilm formed by the corresponding individual species, expressed as a percentage.

$$\text{Inhibition}(\%) = \left[\frac{(OD_1 + OD_2) - OD_3}{(OD_1 + OD_2)}\right] \cdot 100 \tag{1}$$

where OD_1 = the OD of *K. pneumoniae* individual species biofilm; OD_2 = the OD *E. faecalis* individual species biofilm; and OD_3 = the OD of the mixed biofilm.

Quantification of cultivable cells

The number of cultivable cells from disrupted biofilms was obtained by colony counts. In brief, after the pipetting of supernatants, the wells were rinsed once with 1 × PBS to remove non-attached cells. The plates were then sonicated at 40 kHz for 1 min, following the protocol described by Iñiguez-Moreno et al. (2017). Later, the biofilms were scraped with a cell scraper (VWR international) and serially diluted for colony counts. In monomicrobial biofilms, *K. pneumoniae* and *E. faecalis* were plated on Luria Bertani agar (Miller's LB AGAR, Condalab) and BD Columbia agar with 5% Sheep Blood (Becton Dickinson), respectively. In polymicrobial biofilms, aliquots were plated both on selective media MacConkey II agar (Becton Dickinson) for selection of *K. pneumoniae* cells and on Enterococcosel agar (Becton Dickinson) for *E. faecalis*. Agar plates were incubated at 37 °C for 18-24 h. Assays were performed in triplicate.

Competitive index

The competitive index (CI) was calculated according to Macho et al. (2007). Thus, the CI was defined as the *K. pneumoniae/E. faecalis* ratio within the output sample, divided by the corresponding ratio in the inoculum (input), where output and input samples were evaluated after serial dilutions of the sample taken at fixed times and plating onto selective media. CI values were subjected to a Log transformation for normal distribution, and then interpreted as follows: a CI value equal to zero indicates no competition between the two species; a positive CI value indicates a competitive advantage for *K. pneumoniae*, and a negative CI value indicates a competitive advantage for *E. faecalis* (Magalhães et al. 2017).where CI = 0:

$$\mathrm{CI} = \mathrm{Log}\left[\frac{(\mathrm{CFU\ ml^{-1}}\ K.\ pneumoniae)\ \mathrm{output} \cdot (\mathrm{CFU\ ml^{-1}}\ E.\ faecalis)\ \mathrm{input}}{(\mathrm{CFU\ ml^{-1}}\ E.\ faecalis)\ \mathrm{output} \cdot (\mathrm{CFU\ ml^{-1}}\ K.\ pneumoniae)\ \mathrm{input}}\right] \quad (2)$$

equal competition between species; $\mathrm{CI} > 0$: competitive advantage for *K. pneumoniae* and $\mathrm{CI} < 0$: competitive advantage for *E. faecalis*.

Competition in planktonic cultures

The same volume of $\sim 1 \times 10^7$ CFU ml^{-1} of each strain was mixed and incubated at 37 °C with shaking at 180 rpm. Aliquots were taken after specific times (24, 48, and 72 h), serially diluted 10-fold if needed and plated for colony counting as described above. The cell counts were expressed as CFU ml^{-1}. The tests were performed in triplicate. After each cell count, the culture was centrifuged, the supernatant discarded and replaced with fresh medium and the cells were resuspended in the new medium.

Supernatant analysis

Lyophilized cell-free supernatant collection (L-CFS)

E. faecalis supernatants from biofilms were collected after incubation for 24 h. A portion of each supernatant was adjusted with 1 M sodium hydroxide (NaOH) at pH 6.5. The total volume collected was then centrifuged for 15 min at 12,000 ×g (at 4 °C) and passed through a 0.22 µm pore-size filter to remove bacteria and obtain cell-free supernatants (CFS) (Wang et al. 2013). They were then lyophilized in a CHRIST freeze dryer alpha 1-2 LD (Martin Christ Gefriertrocknungsanlagen GmbH) to obtain lyophilized cell-free supernatants (L-CFS).

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) values of L-CFS were determined with the broth microdilution method (CLSI. 2019). Two-fold serial dilutions in Mueller Hinton broth (Condalab) were carried out using round-bottom microtiter plates (Greiner bio-one 96 well, polystyrene, U-bottom). The final volume in each well was 100 µl. Bacterial 0.5 McFarland suspensions were diluted to obtain a final concentration of 5×10^5 CFU ml^{-1} per well. The MIC of DL-lactic acid 85% (w/w), syrup (Sigma Aldrich) was also measured according to the same protocol. In order to avoid evaporation, all plates were covered with adhesive foil lids and incubated under static conditions at 37 °C for 18–24 h and read visually for the absence of turbidity. MIC values were defined as the lowest concentration of L-CFS that inhibited visible growth. The experiments were carried out in triplicate.

Anti-biofilm assays

The inhibition of biofilm formation was assessed by the determination of the minimum biofilm inhibitory concentration (MBIC) according to the procedure described by DosSantos Goncalves et al. (2014) with some modifications. Briefly, overnight cultures of *K. pneumoniae* were diluted to reach a 10^7 CFU ml^{-1} inoculum with fresh TSB supplemented with 1% glucose. Biofilm formation was carried out in polystyrene flat bottomed microtiter plates (Nunc™ Edge 2.0 96-well plate, non-treated, with lid, VWR International). Each well, filled with the corresponding inoculum, contained 1/4 (v/v) of *E. faecalis* supernatant extract at different concentrations. The microtiter plates were incubated at 37 °C for 24 h under static conditions. Each well was rinsed once with sterile 1 × PBS and the remaining biofilms were quantified following the CV staining procedure described above.

To evaluate the effects of L-CFS on pre-formed *K. pneumoniae* biofilms, the minimum biofilm eradication concentration (MBEC) was assessed as follows. After incubation for 24 h at 37 °C under static conditions, each well containing established biofilm was carefully rinsed once with sterile 1 × PBS and treated with L-CFS at different concentrations. The microtiter plates were then incubated at 37 °C for another 24 h and quantified using the CV staining procedure. In both assays, a negative control (culture medium without inoculum) and positive control (culture medium with bacterial inoculum) were provided on each plate.

Both the inhibitory and eradication capacities of DL-lactic acid 85% against *K. pneumoniae* biofilms were also measured following the same protocol. The experiments were carried out in three technical and biological replicates.

Determination of lactic acid

Quantitative detection of lactic acid in cell-free supernatants was performed using the L-lactic acid Kit (BioSystems S.A.). The method is based on lactic acid oxidation. L-lactic acid in the sample can be

measured by spectrophotometry. Measurements were made on the Analyser Y15 (BioSystems S.A.).

$$\text{L-lactate} + \text{NAD}^+ \xrightarrow{\text{LLDH}} \text{pyruvate} + \text{NADH} \quad (3)$$

Biofilm development using lactic acid E. faecalis mutant strains

E. faecalis V583 wt and its mutant strains with deletions in either *ldh-1*, *ldh-2*, or both genes were used to assess the inhibition caused by lactic acid production. Development and quantification of mono- and polymicrobial biofilms by bacterial CFUs counts were performed as described above. The pH of the supernatants was also measured with the pH-Meter BASIC 20 (CRISON INSTRUMENTS S.A.).

Interspecies interaction with pooled human urine

To evaluate the interactions between some of the strains, human urine was collected from six healthy volunteers of both genders who had no history of urinary tract infection. Urinalysis showed normal parameters (glucose, ketones, nitrites, leukocyte esterase, bilirubin, urobilinogen, blood, and proteins). The urine was pooled, filter sterilised and stored at 4 °C. The pH of the urine was 6.5 at the beginning of the analysis. Mono- and polymicrobial biofilms were developed using pooled human urine with or without glucose 1%, and quantified by CFU counts. The CI was also assessed.

Data plotting and statistical analysis

All statistical analyses were performed with GraphPad Prism v8.0.2 software (La Jolla, California, USA). Graphs were created using GraphPad Prism v8.0.2 software and Tableau Software (Seattle, USA). The data are expressed as means ± SD (standard deviation). The percentage inhibition of biofilm formation was evaluated *via* the Student t-test, comparing the OD_{580nm} value of polymicrobial biofilms and the sum of OD_{580nm} of each monomicrobial biofilm. One-way ANOVAs followed by *post hoc* Dunnett's multiple comparisons tests were used to analyse the quantification of the cultivable cells, competition in planktonic cultures, and the anti-biofilm capacities (MBIC and MBEC) by the L-CFS of *E. faecalis*. One-way ANOVA followed by *post hoc* Tukey's multiple comparisons test was used to analyse adhesion to abiotic surfaces. Confirmation of inhibition by lactic acid production using *E. faecalis* V583 wt and its mutants was performed by confidence intervals on the difference between means. Tests with *P* values < 0.05 were considered significant.

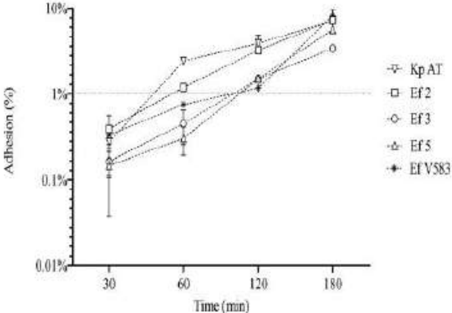

Figure 1. Adhesion to abiotic surface. Adhesion to polystyrene plates was measured at 30, 60, 120, and 180 min. Each point in the curve represents the mean values of adherent cell percentage. Vertical bars correspond to SDs. The strains were considered highly adherent to a surface when the percentage of adherent bacteria was superior to 1 % of the original inoculum. One-way ANOVA showed statistically significant differences when the percentage adherence of *K. pneumoniae* AT after incubation for 60 min was compared with the percentage adherence of all *E. faecalis* strains tested at the same incubation time. ($p < 0.0001$).

Results

Biofilm assays

Adhesion to abiotic surfaces

The time-dependent adhesion to polystyrene plates was measured by conventional plating on solid media (Figures 1 and S1). The number of adhered bacteria increased during the incubation period for all the strains tested. *K. pneumoniae* AT was the most adherent strain with an increase of 2.42% after 60 min incubation compared to the initial inoculum. Among the *E. faecalis* strains, Ef 2 increased adhesion by 1.20% after 60 min incubation, and Ef 3, Ef 5, and Ef V583 reached a percentage of 1.50%, 1.51%, and 1.18%, respectively, after 120 min incubation. One-way ANOVA showed statistically significant differences when comparing the percentage of adhesion of *K. pneumoniae* AT after 60 min incubation with the percentages of adhesion of all *E. faecalis* strains tested at the same incubation time ($p < 0.0001$).

Percentage inhibition of biofilm formation

When assessing the interaction between the two pathogens within the biofilm, the reduction in the total biomass of the polymicrobial biofilms of *K. pneumoniae* and *E. faecalis* compared with the sum of the total biomass of the monomicrobial biofilms was

statistically significant in all the comparisons ($p < 0.001$) (Table 2). The same effect was observed for the other *K. pneumoniae* strains (Table S1). These results indicate that the co-cultivation of *K. pneumoniae* and *E. faecalis* in a polymicrobial biofilm significantly impaired their development compared with monomicrobial biofilms.

Quantification of cultivable cells

The results of the cultivable bacterial quantification after polymicrobial biofilm growth at different points in time are shown in Figures 2 and S2. In general, the cell numbers of *K. pneumoniae* in the polymicrobial biofilms decreased compared with the monomicrobial biofilms. *E. faecalis* showed similar growth in the polymicrobial biofilms compared with the monomicrobial and was the predominant species in co-culture with *K. pneumoniae*. This predominance was not statistically significant after incubation for 30 min, 1, 2, 3, 4, and 8 h. A statistically significant reduction in CFUs was observed in *K. pneumoniae* after 24, 48, and 72 h ($p < 0.001$). Differences observed in *K. pneumoniae* colony counts also depend on the *E. faecalis* strain used in the polymicrobial biofilm and may be the result of nutrient competition and the difference in growth rates of the strains. Similarly, inherent characteristics of the individual strains, such as virulence factors and their expression as well as specific physical interactions or the exchange of metabolites between the strains, could also cause these differences.

Table 2. Reduction in polymicrobial biofilm formation (% ± SD) compared with monomicrobial biofilms.

	24 h	48 h	72 h
Kp AT + Ef 2	45.33 ± 2.4	54.75 ± 0.60	46.32 ± 2.2
Kp AT + Ef 3	61.21 ± 0.97	70.29 ± 0.65	56.27 ± 0.58
Kp AT + Ef 5	55.84 ± 9.4	54.35 ± 10	53.32 ± 6.0
Kp AT + Ef V583	54.99 ± 7.8	56.61 ± 3.2	54.45 ± 9.7

Competitive index

The CI values allow comparison of the differences between the growth curves of polymicrobial biofilms and help to explain which of the pathogens present in the co-culture showed a predominant behaviour within the biofilm. Negative CI values in polymicrobial biofilms were observed during most of the incubation periods tested (Figures 3 and S3), confirming the results of the colony counts. This indicates a clear advantage of *E. faecalis* over *K. pneumoniae*.

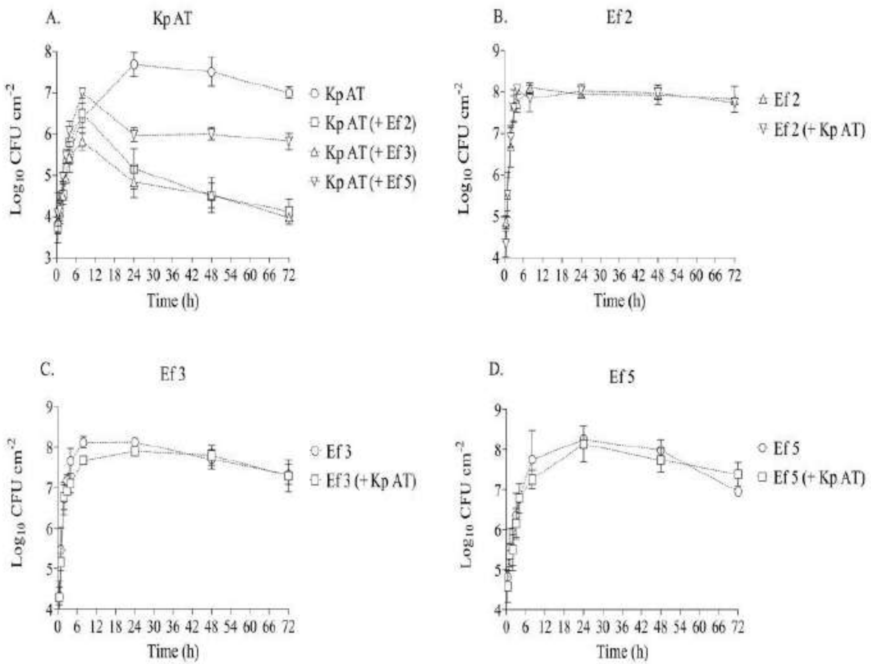

Figure 2. Cultivable cells quantification. Each point in the curve represents mean values of $\log_{10}$ CFU cm^{-2} recovered from biofilm after incubation for 30 min, 1, 2, 3, 4, 8, 24, 48, and 72 h; vertical bars correspond to SDs. At 24, 48, and 72 h, statistically significant differences were observed in the colony counts of *K. pneumoniae* when compared with monomicrobial biofilm ($p < 0.001$, One-way ANOVAs followed by *post hoc* Dunnett's multiple comparisons tests). (A) *K. pneumoniae* AT monomicrobial biofilm and co-cultured with *E. faecalis* 2, 3 or 5. (B) *E. faecalis* 2 monomicrobial biofilm and co-cultured with *K. pneumoniae* AT. (C) *E. faecalis* 3 monomicrobial biofilm and co-cultured with Kp AT. (D) *E. faecalis* 5 monomicrobial biofilm and co-cultured with *K. pneumoniae* AT.

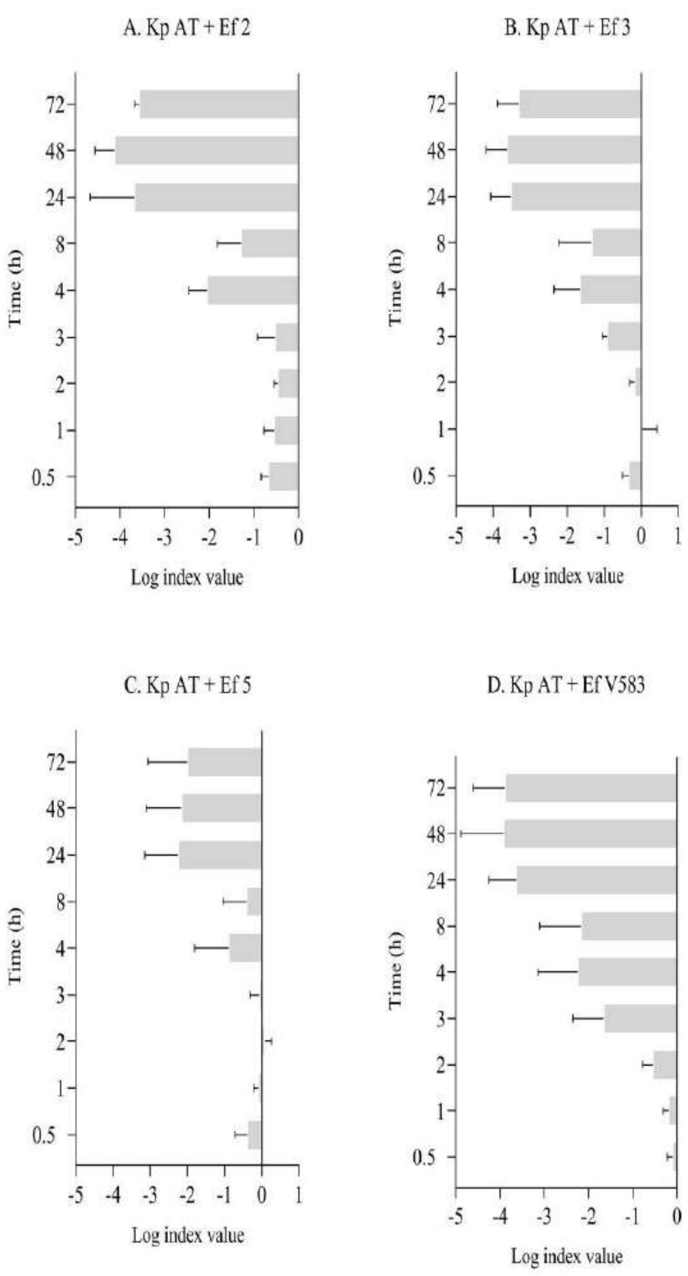

Figure 3. Competitive index (CI). Each bar in the graph represents mean values of Log index; horizontal bars correspond to SDs. A CI value equal to zero indicates no competition between the two species; a positive CI value indicates a competitive advantage for *K. pneumoniae*, and a negative CI value indicates a competitive advantage for *E. faecalis*. (A) CI of *K. pneumoniae* AT co-cultured with *E. faecalis* 2. (B) CI of *K. pneumoniae* AT co-cultured with *E. faecalis* 3. (C) CI of *K. pneumoniae* AT co-cultured with *E. faecalis* 5. (D) CI of *K. pneumoniae* AT co-cultured with *E. faecalis* V583.

Competition in planktonic cultures

Although a reduction in the CFUs of *K. pneumoniae* was observed when co-cultured with different *E. faecalis* strains in biofilm, a statistically significant reduction in CFUs was not observed in planktonic co-cultures compared with monocultures in any of the species involved and for any of the time points tested ($p > 0.05$). Therefore, neutral interactions between the species were obvious in the planktonic state (Figure S4).

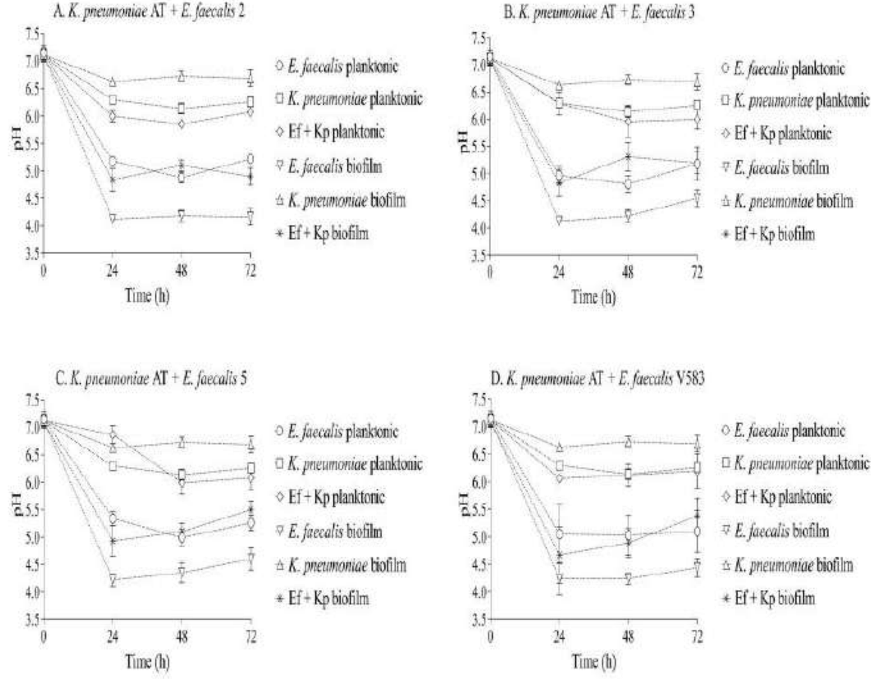

Figure 4. pH measurement. Each point in the curve represents the mean pH values of supernatants recovered after incubation for 24, 48, and 72 h; vertical bars correspond to SDs. (A) *K. pneumoniae* AT and *E. faecalis* 2. (B) *K. pneumoniae* AT and *E. faecalis* 3. (C) *K. pneumoniae* AT and *E. faecalis* 5. (D) *K. pneumoniae* AT and *E. faecalis* V583.

Supernatant analysis

pH measurement

The pH values of the supernatants of mono- and polymicrobial cultures (biofilms and planktonic) were measured during the entire incubation period with the pH-Meter Basic 20 (CRISON INSTRUMENTS S.A.). A pH decrease through the incubation time was observed in both the mono- and co-cultures (Figure 4). For biofilms, it should be noted that the pH of the *E. faecalis* supernatant was lower in the monomicrobial cultures than in the polymicrobial cultures. Nevertheless, the pH value decreased sufficiently to inhibit the growth of *K. pneumoniae* in co-culture (Figure 2). Conversely, the pH in polymicrobial planktonic cultures had not been lowered sufficiently to affect the growth of *K. pneumoniae*, and the colony counts of the different strains had not been affected.

Antimicrobial and antibiofilm effects

All non-pH-adjusted L-CFS collected from *E. faecalis* biofilms showed antimicrobial and antibiofilm activity against *K. pneumoniae*. Thus, the MIC value was 32 mg ml^{-1}. The minimum biofilm inhibitory concentration (MBIC), defined as the last well in which no visible growth was observed after incubation in the presence of biofilm and antimicrobial agents (LaPlante and Mermel 2009), was 64 mg ml^{-1} (Figures 5a and S5). The minimum biofilm eradication concentration (MBEC), defined as the lowest concentration required by an antimicrobial agent to eradicate the biofilm (Perumal and Mahmud 2013), was 256 mg ml^{-1}, although no complete eradication of the mature biofilm was observed in *K. pneumoniae* AT (Figures 5b and S6).

MBIC and MBEC were measured in three biological and technical replicates and were statistically significant compared with the control *(K. pneumoniae* biofilm without L-CFS) ($p < 0.001$). No antimicrobial or anti-biofilm activity was observed when the pH of L-CFS collected from biofilms was adjusted to a pH of 6.5 with 1 M sodium hydroxide (NaOH). The antibacterial and anti-biofilm activity of commercial lactic acid was also demonstrated against *K. pneumoniae*, being the MIC = 1.25 mg ml^{-1}, MBIC = 4 mg ml^{-1}, and MBEC > 256 mg ml^{-1}.

Determination of lactic acid in supernatants

To confirm that the decrease in pH was due to the production of organic acids, the lactic acid concentration of the supernatants collected from biofilms was measured. A significant lactic acid concentration was found in the supernatants (Tables 3 and S2), which can confer the observed antibacterial and anti-biofilm activities against *K. pneumoniae*. These results are

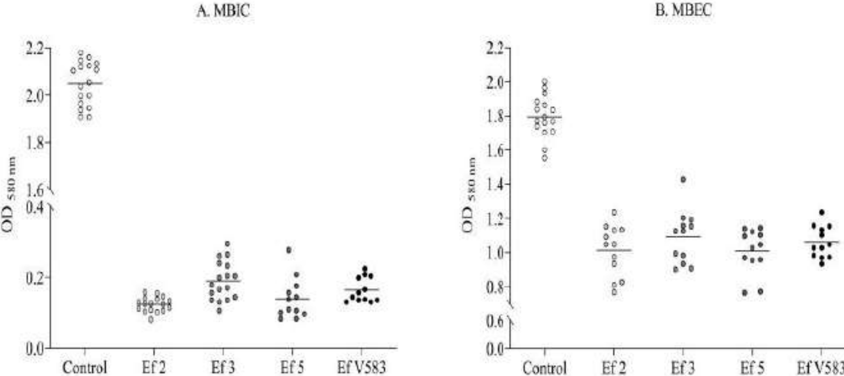

Figure 5. (A) Scatter dot plot showing the anti-biofilm effect produced by L-CFS collected from *E. faecalis* biofilm. Each dot in the graph represents an individual datum. Horizontal lines represent mean values of OD_{580nm}. Statistical significance was observed in all the measures in comparison with the controls (*K. pneumoniae* biofilm without L-CFS) ($p < 0.001$ One-way ANOVAs followed by *post hoc* Dunnett's multiple comparisons tests). (B) Scatter dot plot showing partial eradication of preformed *K. pneumoniae* biofilm using the L-CFS obtained from *E. faecalis* biofilms. Each dot in the graph represents an individual datum. Horizontal lines represent mean values of OD_{580nm}. In all comparisons, statistical significance was observed in comparison with the controls (*K. pneumoniae* biofilm without treatment) ($p < 0.001$, One-way ANOVAs followed by *post hoc* Dunnett's multiple comparisons tests).

Table 3. pH values and lactic acid concentration (g l^{-1}) of supernatants collected from biofilms after 24 h incubation, expressed as means ± SDs.

	Biofilm supernatants	
	pH	Lactic acid concentration (g l-1)
Ef 2	4.12 ± 0.04	2.44 ± 0.02
Ef 3	4.13 ± 0.08	2.39 ± 0.02
Ef 5	4.22 ± 0.12	2.35 ± 0.03
Ef V583	4.24 ± 0.29	2.28 ± 0.05
Ef 2 + Kp AT	4.84 ± 0.21	1.91 ± 0.02
Ef 3 + Kp AT	4.83 ± 0.25	1.91 ± 0.03
Ef 5 + Kp AT	4.93 ± 0.28	1.88 ± 0.04
Ef V583 + Kp AT	4.80 ± 0.69	1.81 ± 0.06

consistent with MIC values obtained with commercial lactic acid, where a concentration of 1.25 mg ml^{-1} inhibited the growth of *K. pneumoniae*. In addition to the pH value measured, the lactic acid of the *E. faecalis* supernatant was higher in the monomicrobial cultures than in the polymicrobial cultures. The two species with different growth rates compete for nutrients in polymicrobial cultures. Therefore, *E. faecalis* has not the same nutrient availability to produce the same level of lactic acid as in monomicrobial biofilms.

K. pneumoniae *biofilm development at different pH conditions*

To define the influence of pH on the growth and subsequent biofilm development of *K. pneumoniae*, TSB was adjusted with 1 M NaOH at pH values ranging from 3.5 to 7.0, with intervals of 0.5. The biofilms were prepared according to the protocol of biofilm development and quantification and then incubated under static conditions at 37 °C for 24 h. After

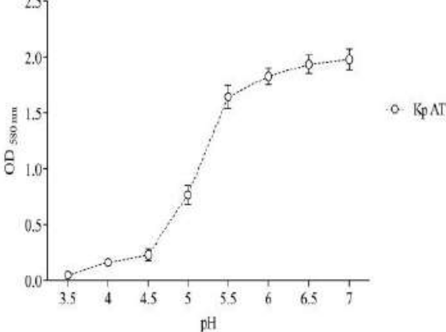

Figure 6. *K. pneumoniae* AT biofilm development at different pH values. TSB medium was adjusted to different pH ranging from 3.5 to 7.0. Biofilms were established and incubated in static conditions at 37 °C for 24 h. After incubation, biofilm production was quantified using CV staining. Each point in the graph shows the mean of $OD5_{80nm}$ measurements after crystal violet staining. Vertical bars correspond to SDs.

incubation, biofilm production was quantified by CV staining. The results of $OD_{580\,nm}$ showed that the lowest pH value at which *K. pneumoniae* AT can form biofilms was 5.0, where 7.0 is the optimal pH value for the development of a strong biofilm. This condition corresponds to the pH value used in conventional culture media (Figures 6 and S7).

Biofilm development using lactic acid E. faecalis *mutant strains*

E. faecalis possesses two cytosolic L - (+) - lactate dehydrogenases encoded by the genes *ldh-1* and *ldh-2*. Most of the activity is associated with LDH-1, and LDH-2 plays only a minor role (Fatima Rana et al. 2013). Therefore, polymicrobial biofilms formed by *E.*

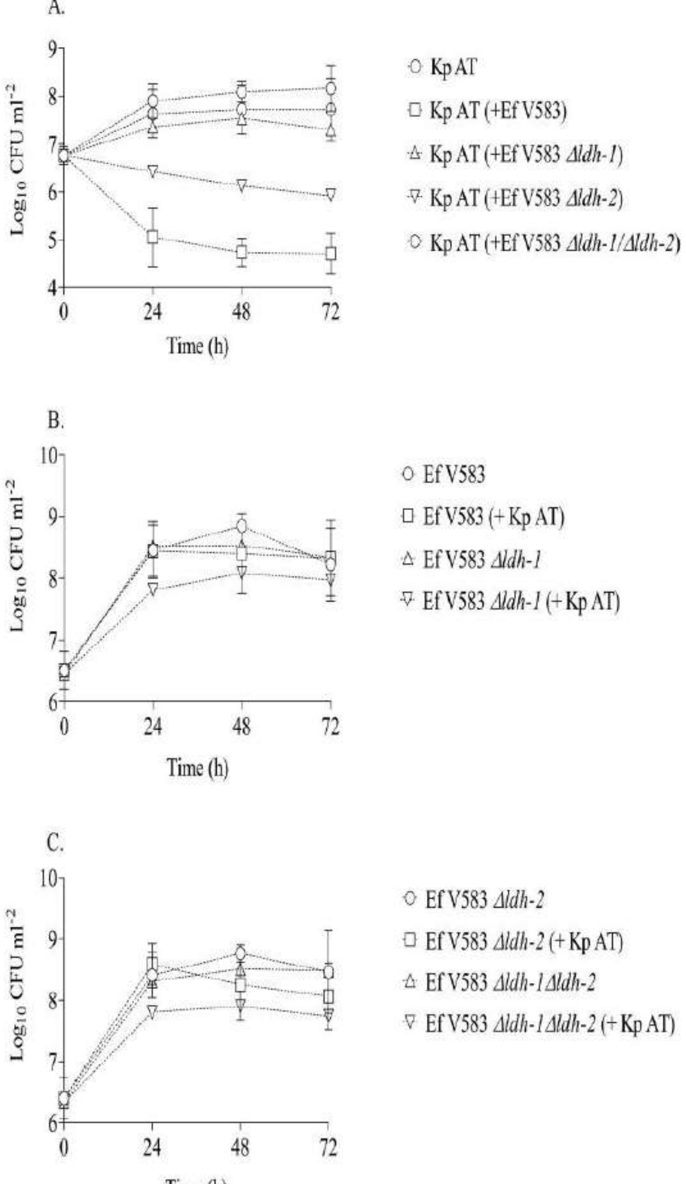

Figure 7. Biofilm development using lactic acid *E. faecalis* mutant strains. *E. faecalis* V583 wt, *Δldh-1*, *Δldh-2*, and *Δldh-1/Δldh-2* double mutants were used to probe the influence of lactic acid production on the growth inhibition of *K. pneumoniae* in polymicrobial biofilms. Each point in the curve represents mean values of $\log_{10}$ CFU cm^{-2} recovered from biofilm after incubation for 24, 48, and 72 h; vertical bars correspond to SDs. Statistically significant differences were observed in colony counts of *K. pneumoniae* when compared with monomicrobial biofilm, when the polymicrobial biofilm was formed by *E. faecalis* V583 wt and V583 *Δldh-2* ($p < 0.001$, One-way ANOVAs followed by post hoc Dunnett's multiple comparisons tests). No statistical significance was observed when the co-culture was formed with *E. faecalis* V583 *Δldh-1*, and V583 *Δldh-1/Δldh-2* double mutant. (A) *K. pneumoniae* AT monomicrobial biofilm and co-cultured with *E. faecalis* V583 wt, *Δldh-1*, *Δldh-2*, and *Δldh-1/Δldh-2* double mutant. (B) *E. faecalis* V583 and V583 *Δldh-1* monomicrobial biofilms and co-cultured with *K. pneumoniae* AT. (C) *E. faecalis* V583 *Δldh-2* and V583 *Δldh-1/Δldh-2* double mutant monomicrobial biofilms and co-cultured with *K. pneumoniae* AT.

faecalis V583 wt or V583 *Δldh-2* showed the same inhibitory effect against *K. pneumoniae* as previously observed in the other clinical strains of *E. faecalis* tested in this study. However, when *E. faecalis* V583 *Δldh-1* or *Δldh-1/Δldh-2* double mutant were analysed, the colony counts of *K. pneumoniae* were not statistically affected compared with monocultures (Figure 7). The confidence intervals for the differences

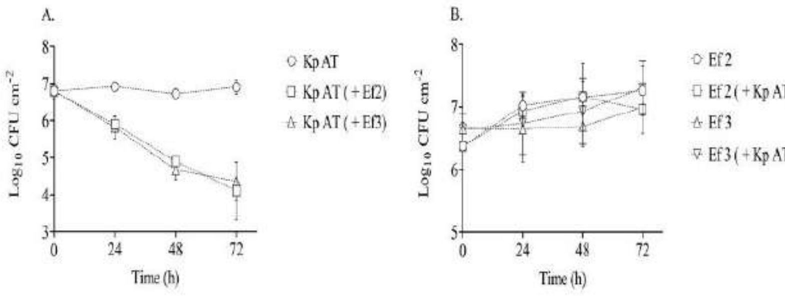

Figure 8. Cultivable cell quantification using pooled human urine. Each point in the curve represents mean values of $\log_{10}$ CFU cm^{-2} recovered from biofilm after incubation for 4, 48, and 72 h; vertical bars correspond to SDs. At 24, 48, and 72 h, statistically significant differences were observed in the colony counts of *K. pneumoniae* when compared with the monomicrobial biofilm ($p < 0.001$, One-way ANOVAs followed by *post hoc* Dunnett's multiple comparisons tests). (A) *K. pneumoniae* AT monomicrobial biofilm and co-cultured with *E. faecalis* 2 or 3. (B) *E. faecalis* 2 and *E. faecalis* 3 monomicrobial biofilms and co-cultured with *K. pneumoniae* AT.

between the mean values showed statistically significant differences between the mean values of *K. pneumoniae* AT monomicrobial and *K. pneumoniae* AT co-cultured with *E. faecalis* V583 wt or *E. faecalis* V583 Δ*ldh-2*. The difference between the mean values of *K. pneumoniae* AT monomicrobial and *K.pneumoniae* AT co-cultured with *E. faecalis* V583 Δ*ldh-1* or *E. faecalis* V583 Δ*ldh-1*/Δ*ldh-2* was not statistically significant. The same effect was observed with the other *K. pneumoniae* strains (Figure S8). The CI showed an advantage of *E. faecalis* over *K.pneumoniae* in all the strains used, but the difference in values obtained using *E. faecalis* V583 or *E. faecalis* V583 Δ*ldh-2* (-3.63 and −2.52 after 24 h of incubation, respectively) was higher than the values obtained when *E. faecalis* V583 Δ*ldh-1* or *E. faecalis* V583 Δ*ldh-1*/Δ*ldh-2* were used in polymicrobial biofilms (-0.74 and −0.59 respectively, after incubation for 24 h). In the same way, the pH reduction in the polymicrobial cultures with *E. faecalis* V583 or *E. faecalis* V583 Δ*ldh-2* was sufficient to inhibit the growth of *K. pneumoniae*. Although the pH reduction could be achieved by other organic acids produced, the loss of lactic acid production in these *E. faecalis* mutant strains (V583 Δ*ldh-1* or Δ*ldh-1*/Δ*ldh-2* double mutant) made these values not as low as the wt V583 or the V583 Δ*ldh-2* strain, resulting in modest changes in *K. pneumoniae* growth (Table S3).

Interspecies interaction with pooled human urine

The urine conditions of diabetics and non-diabetics were simulated with pooled human urine with and without glucose. Neutral interactions between the strains were found when using urine without glucose. However, the inhibitory effects of *E. faecalis* on *K. pneumoniae* were observed when pooled human urine was supplemented with 1% glucose – similar to the use of glucose-supplemented TSB (Figures 8 and S9). The reduction in the CFUs of *K. pneumoniae* was statistically significant after incubation for 24, 48, and 72 h ($p < 0.001$). Negative CI values were observed during all the incubation periods tested, confirming the results of colony counts.

Discussion

It is well known that the interactions established in polymicrobial biofilms imply cell-cell communication, typically *via* quorum sensing (Thornhill and McLean 2018). Compared with monomicrobial biofilms, these interactions can promote a synergism, in which participating species cooperate with each other by increasing biofilm formation and, thus, their resistance to antibiotics (Schwering et al. 2013; Makovcova et al. 2017). However, these interactions may lead to an advantage for one of the participating species, based on nutrient competition or by inhibiting the proper growth of their counterparts, a mechanism known as antagonism (Harrison 2007). Therefore, the co-culture of different bacteria in the biofilm state can lead to an increase or a decrease in their biomass. The third scenario is one in which neither synergism nor antagonism can be detected among the species involved. Therefore, in this case, their interaction is considered neutral.

Considering that *K. pneumoniae* and *E. faecalis* are common uropathogens and that biofilm formation is an important feature in their pathogenesis, the study of their interactions between species within biofilms seems to be mandatory. This approach could help to identify possible targets or new antimicrobial compounds, mainly produced by dominant strains, with therapeutic effects. However, research on this specific interaction is still scarce.

According to the present study, at the time the CV assay and the percentage of inhibition was performed, the sum of their separate monomicrobial biomasses did not correlate with the total biomass of the polymicrobial biofilms, which was often much lower. Therefore, these results indicate a CI between the species involved, which is also supported by their quantification on agar plates, where the dominance of *E. faecalis* over *K. pneumoniae* was observed. In polymicrobial biofilms, the CFU cm^{-2} values of *E. faecalis* remained nearly unchanged overall tested periods, whereas the colony counts of *K. pneumoniae* decreased over time in contrast with the monomicrobial biofilm. In the same way, the negative CI values obtained over time indicate an inhibitory effect of all *E. faecalis* against *K. pneumoniae*, indicating a competitive rather than cooperative interaction between the species. It is important to note that this competitive interaction was not observed in the planktonic state, although the nutrient content of the growth medium, pH, and temperature were initially the same in both experiments. This was because the oxygen availability between the cells in the planktonic state and the biofilm state differs. Thus, as observed in the biofilm assay, limited aeration lead to an increase in lactic acid production by *E. faecalis* compared with planktonic cultures.

In a previous study by Galván et al. (2016) neutral interactions between *K. pneumoniae* - *E. faecalis* regarding biofilm formation were observed and the number of viable cells was similar when mixed and pure cultures were compared. The present results contrast with this earlier work and show a decrease in the biofilm population of *K. pneumoniae* and an advantage of *E. faecalis* in biofilm co-culture. These differences observed between the studies could be attributed to the use of an artificial urine medium without glucose, which, when tested in the authors' laboratory, did not allow adequate growth of the *E. faecalis* strains tested. Therefore, pooled human urine supplemented with glucose was used, observing the same inhibitory effect as when using TSB broth. It should be noted that, as observed by Galván et al. (2016), neutral interspecies interactions were observed when pooled human urine without glucose was used, which makes a difference in inter-species interactions that can occur in diabetics and non-diabetics. In patients with CAUTI and risk factors such as diabetes, less virulent pathogens than Enterobacteriaceae, such as *Candida* spp. and *Enterococcus* spp., become more prevalent (Tandogdu and Wagenlehner 2016), and other studies have shown that urinary tract infections caused by *Enterococcus* species are often polymicrobial (Giannakopoulos et al. 2019), which confirm the present findings. Although the data obtained in this study have the limitation of not using a continuous flow system, future research should focus on a comparison between the static and dynamic models.

Nadell et al. (2016) confirmed that when several strains and species come into contact with others, the predominance of the most competitive phenotypes is expected, as an act of natural selection to favour genetic lines that may be more helpful for them than for the others. In this way, *K. pneumoniae*, although it has a shorter generation time than *E. faecalis*, is not able to maintain dominance in the biofilm. Schluter et al. (2015) found that in polymicrobial biofilms the most adherent genotype predominates, but the results obtained suggest that although *K. pneumoniae* AT has a stronger adhesion capacity in monomicrobial biofilms than *E. faecalis*, this characteristic is not decisive for the further development of the polymicrobial biofilm with *E. faecalis*, where the production of metabolites such as lactic acid influences the growth of *K. pneumoniae*. Therefore, not only adhesion to the abiotic surface but also the production of inhibitors should be considered when analysing these interactions.

Several bacteria produce different compounds that interfere with the growth of their opponents, such as hydrogen peroxide, various organic acids or bacteriocins (Mariam et al. 2017). Bacteriocins are stable at different pH concentrations and have different biological and physico-chemical properties, some of which are related to their ability to eradicate biofilms (Mathur et al. 2018). In some cases, however, one of the species involved produces hydrogen peroxide or organic acids as metabolic waste, resulting in a change in the pH of the surrounding medium that damages the other species growing in the biofilm (Makovcova et al. 2017). The producing strain might therefore benefit from its waste product itself or not be affected by it, but accidentally it might interfere with the normal growth of the other species (Nadell et al. 2016). For example, vaginal *Lactobacilli* spp. produce lactic acid which causes a lowering of the pH in the environment and interferes with the growth of species such as *Neisseria gonorrhoeae* (Graver and Wade 2011). *E. faecalis* is also considered a lactic acid bacterium, and some of the mechanisms involved in the inhibition of various pathogens include competition for nutrients, production of various organic acids and secretion of antimicrobial substances (Mariam et al.

2017). Lactic acid is the main product of *Enterococcus* fermentation under conditions of excess glucose and limited oxygen content. It is produced by the reduction of pyruvate for the regeneration of NAD^+ for ongoing glycolysis and *Enterococcus* is considered the most important of all lactic acid bacteria (Ramsey et al. 2014). Certain strains, however, produce ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin (DeVuyst & Leroy 2007).

Antibacterial and antibiofilm effects were also observed in the evaluation of the activity of L-CFS extracted from *E. faecalis* biofilms against *K. pneumoniae*. Since the inhibitory effect was observed only in the testing of non-pH-adjusted L-CFS, it was probably caused by the production of organic acids and may not be related to bacteriocin production. Thus, when lactic acid in supernatants was measured, the concentration was sufficient to inhibit the growth and biofilm development of *K. pneumoniae*, which was also supported by the commercial lactic acid MIC and MBIC results.

E. faecalis has been found to inhibit the growth of other bacteria in various environments. In root canals, *Streptococcus gordonii* was completely inhibited when co-cultured with *E. faecalis* (Gao et al. 2016). Another study showed that the presence of *E. faecalis* limited the presence of *Listeria monocytogenes* in polymicrobial biofilms at 39 °C due to competition for nutrients and the production of toxic metabolites (DaSilva Fernandes et al. 2015). Studies by Alakomi et al. (2000) confirmed that 5 mM (pH 4.0) lactic acid caused the permeabilisation of Gram-negative bacteria by breaking the outer membrane. The average pH value found in the biofilm supernatants of *E. faecalis* after 24 h was 4.18 and the lactic acid concentration was 2.37 g l^{-1}. In addition, when testing the ability of *K. pneumoniae* to form a biofilm in a pH range between 3.5 and 7.0, biofilm biomass production increased together with the pH of the medium, with pH 7.0 being found to be the most favourable condition for biofilm formation. On the other hand, bacteria lose this ability at pH values below 5.0. Hoštacká et al. (2010) also demonstrated the effect of pH on biofilm production and found that biofilm production by *K. pneumoniae* increased by 151–319% at pH 8.5, and 111–177% at pH 7.5 compared with the biofilm formed at pH 5.5. Furthermore, the analysis carried out with *E. faecalis* V583 wt and V583 mutant strains with deletions in *ldh-1*, *ldh-2*, or both genes, also confirms the hypothesis of inhibition caused by lactic acid production. The inhibition was found when the polymicrobial biofilms of *E. faecalis* V583 wt or the *E. faecalis V583 Δldh2* strains were formed. On the other hand, no statistically significant decrease in colony counts of *K. pneumoniae* when *E. faecalis V583 Δldh-1* or *Δldh-1/Δldh-2* mutant strains were involved. This is because most of the lactic acid production is conferred to the *ldh-1* gene, with *ldh-2* playing a minor role in this process (Jönsson et al. 2009; Fatima Rana et al. 2013), suggesting that the antibiofilm effect of *E. faecalis* on *K. pneumoniae* is mainly due to the production of lactic acid and the resulting reduction in pH. This suggests that in CAUTIs of diabetic patients, lactic acid production may give *E. faecalis* an advantage over *K. pneumoniae* or other species, as some *E. faecalis* strains are resistant and can adapt to different pH ranges growing under highly acidic conditions (pH 2.9) (Rince et al. 2000; Mubarak and Soraya 2018). Furthermore, it is known that growth inhibition of various Gram-negative pathogens occurs in urine at pH ≤ 5 (Kaye 1968). Various researchers have explained how some lactic acid bacteria can be used as candidates for the development of probiotic microorganisms that could inhibit uropathogens. Probiotics have been studied in adult females and children, and have been used for urogenital tract health (Reid and Bruce 2001; Lim et al. 2009; Akgül and Karakan 2018). *E. faecalis* has been proposed as a probiotic due to its adhesion to intestinal cells and the strengthening ability of the epithelial barrier (Baccouri et al. 2019). Furthermore, a study by Manohar et al. (2020) found *Enterococcus* spp. on practically all catheters investigated regardless of the duration, but not in relation to UTI development during the follow-up, which could provide information on the use of *Enterococcus* as a uropathogenic inhibitor due to lactic acid production during adhesion to catheters, avoiding their adhesion by competition, or by reducing the available nutrients. This should be further explored in future research. All these results lead the authors to continuing the study of potential lactic acid bacteria as a biocontrol agent to combat the problematic development of antibiotic resistance and, in this case, against biofilm formation on indwelling devices associated with urinary tract infections.

In conclusion, *K. pneumoniae* and *E. faecalis* were found to interact competitively when cultivated in biofilms in a high glucose environment. Both microorganisms produced more biomass in monomicrobial than in polymicrobial biofilms. *E. faecalis* showed inhibitory activity against *K. pneumoniae* and modified the pH as a result of lactic acid production, which has harmful effects on *K. pneumoniae*, without affecting its own growth.

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Disclosure statement

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Paper 2. Transposon Insertion in the *purL* Gene Induces Biofilm Depletion in *Escherichia coli* ATCC 25922

Authors: Virginio Cepas, Victoria Ballén, Yaiza Gabasa, Miriam Ramírez, Yuly López and Sara Mª Soto

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Hypothesis: The transposon insertion sequencing method could facilitate the discovery of new genes involved in *E. coli* biofilm formation that could be considered new therapeutic targets.

Objectives: To identify new genes involved in biofilm formation in *E. coli* by transcriptomic and proteomic analysis of isogenic mutants generated by transposition.

Materials and methods: A total of 2,578 *E. coli* mutants were generated by transposon insertion, of which 536 were analysed in this study. Of these, 116 showed altered biofilm rates compared to the wt strain (ATCC 25922), and 20 were classified in the low biofilm formation group. We performed phenotypic characterisation of the mutants (growth curves, congo red (CR), swimming, and haemagglutination assays) to exclude mutants with genes previously described in biofilm formation. Two mutants with the most significant loss of ability to form biofilm were selected for DNA sequencing. After that, Tn263 mutant, classified as low biofilm former (LF) compared to the wt strain (ATCC 25922), showed an interruption in the *purL* gene, involved in the de novo purine biosynthesis. The mutant strain was investigated using proteome and transcriptome analyses.

Moreover, a *purL* knockout (Δ*purL::cat*) was generated to understand the relationship between this gene and the process of biofilm formation. Finally, the protein-coding genes corresponding to the proteins previously identified by SDS-PAGE and mass spectrometry were analysed by qRT-PCR between Tn263, the wt strain, three biofilm-forming and three non-biofilm-forming clinical isolates.

Results: DNA sequence analysis of the mutant strain Tn263 revealed that the transposon was inserted in the N-terminal region of the *purL* gene, which is involved in *de novo* purine biosynthesis. As observed with Tn263, the *ΔpurL::cat* knockout strain was unable to form a biofilm in M63 broth. However, when the medium was supplemented with inosine (50 μg/mL), both the *ΔpurL::cat* knockout strain and the Tn263 mutant regained the ability to form biofilms. Furthermore, the knockout strain also recovered the ability to form biofilms after complementation of the *purL* gene with the pGEM®-T vector. Proteomic analysis revealed 13 proteins with statistically significant differences in expression between the wt strain and the Tn263 mutant strain. At the transcriptomic level, we observed changes in *groL, dnaK, adhE, lptD, cysJ, pta, ilvC, elbB* and *ptsI* in the Tn263 mutant compared to the wt strain. In addition, two genes, *adhE* and *ptsI*, showed significant upregulation in the non-biofilm producers compared to the biofilm producer strains. However, it was impossible to establish a relationship between the transcriptome rates in the mutant strains and the clinical strains.

Conclusions: A mutation in the *purL* gene causes a defective biofilm formation associated with the inability to form curli fimbriae, suggesting that this gene is essential for biofilm formation in *E. coli*. Identifying the *purL* gene as a target would help to understand the mechanisms of biofilm production and to develop new antibiofilm treatments against biofilm-related infections caused by this microorganism.

Article

Transposon Insertion in the *purL* Gene Induces Biofilm Depletion in *Escherichia coli* ATCC 25922

Virginio Cepas †, Victoria Ballén †, Yaiza Gabasa, Miriam Ramírez, Yuly López * and Sara Mª Soto *

ISGlobal, Hospital Clínic—Universitat de Barcelona, 08036 Barcelona, Spain; virginio.cepas@isglobal.org (V.C.); victoria.ballen@isglobal.org (V.B.); yaiza.gabasa@isglobal.org (Y.G.); miriam.ramirez@isglobal.org (M.R.)

* Correspondence: yuly.lopez@isglobal.org (Y.L.); sara.soto@isglobal.org (S.M.S.)

† These authors contributed equally to this work.

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Abstract: Current *Escherichia coli* antibiofilm treatments comprise a combination of antibiotics commonly used against planktonic cells, leading to treatment failure. A better understanding of the genes involved in biofilm formation could facilitate the development of efficient and specific new antibiofilm treatments. A total of 2578 *E. coli* mutants were generated by transposon insertion, of which 536 were analysed in this study. After sequencing, Tn263 mutant, classified as low biofilm-former (LF) compared to the wild-type (wt) strain (ATCC 25922), showed an interruption in the *purL* gene, involved in the de novo purine biosynthesis pathway. To elucidate the role of *purL* in biofilm formation, a knockout was generated showing reduced production of curli fibres, leading to an impaired biofilm formation. These conditions were restored by complementation of the strain or addition of exogenous inosine. Proteomic and transcriptional analyses were performed to characterise the differences caused by *purL* alterations. Thirteen proteins were altered compared to wt. The corresponding genes were analysed by qRT-PCR not only in the Tn263 and wt, but also in clinical strains with different biofilm activity. Overall, this study suggests that *purL* is essential for biofilm formation in *E. coli* and can be considered as a potential antibiofilm target.

Keywords: biofilm; *E. coli*; *purL*; transposon insertion; curli fibers

1. Introduction

Escherichia coli is a well-characterised microorganism frequently used as a laboratory model and in industrial microbiology [1]. In the human body, most gut-resident *E. coli* prevent colonisation by pathogenic bacteria and favour the host by producing vitamin K and B12, which are essential during the blood coagulation process and the formation of red blood cells, respectively [2–4]. However, some *E. coli* can also cause intestinal or extraintestinal infections such as urinary tract infections (UTIs), meningitis, and neonatal sepsis [5–9]. *E. coli* grows as free-living cells or biofilm-forming communities. Microbial biofilms are well-organized communities where microorganisms live embedded in a self-produced extracellular polymeric substance (EPS) which protects against adverse environmental conditions [6]. Biofilms are ubiquitous, being found attached to biological or inert surfaces in diverse ecological niches. In addition, *E. coli* biofilms cause several medical device-related infections, including joint infections, intravascular catheter infections, and catheter-associated urinary tract infections (CAUTI) [6,10,11]. Unfortunately, biofilm-forming organisms have an increased tolerance to antibiotics due to the reduced penetration of the antibiotic through the biofilm extracellular matrix (ECM) [12,13]. Likewise, the bacteria within the biofilms evade the immune system through various defence mechanisms, such as by avoiding the complement system and phagocytosis or by acting as a physical barrier [12]. Therefore, there is an unmet need to elucidate new ways to treat biofilm-related infections. In this

sense, to understand the molecular mechanisms underlying biofilm formation, it is essential to develop efficient new clinical treatments. Several studies have described the mechanisms and gene regulation involved during the three general phases of biofilm formation: attachment, development, and dispersal [8,14–17]. For example, some of the well-characterised virulence factor genes among *E. coli* strains during the initial stages include type 1 fimbriae or pili, curli fimbriae, and flagella. Specifically, disruption in *fim* genes (*fimA* and *fimH*, that encode type-1 fimbrial protein A chain and type 1 fimbrin D-mannose specific adhesion, respectively) reduces initial attachment to abiotic surfaces and biofilm production [18]. Curli fimbriae, encoded by the *csgBAC* operon, provide initial adhesion to abiotic surfaces and promote cell aggregation [19]. Thus, alteration of the *csgA* gene triggers reduced biofilm formation due to a decreased production of the main curli protein CsgA subunit [20]. In addition, alterations in genes involved in motility (*fli*, *flh*, *mot*, and *che* alleles) also decrease biofilm formation [5]. On the other hand, the quorum sensing (QS) system plays a critical role in microbial biofilms during development and dispersal stages.

Despite the current knowledge about these genes, the investigation of new essential genes/proteins during biofilm formation could elucidate unknown candidate genes with a higher potential as antibiofilm targets. In the present study we focus on protein expression and the comparison of gene transcription between an *E. coli* wt strain and the isogenic defective biofilm mutants (Tn) produced by transposon insertion. Taking this into account, the aim of this work was to identify new genes involved in biofilm formation in *E. coli* using phenotypic and molecular tools.

2. Materials and Methods

2.1. Culture of E. coli and Electrocompetent Cells

The *E. coli* ATCC 25922 reference strain, used as the wt strain in this study, was grown for 24 h at 37 °C in Luria Bertani (LB) agar (Miller's LB AGAR, Condalab, Madrid, Spain). Electrocompetent cells were made after the overnight culture of a single colony in LB broth (Miller's LB Broth, Condalab) at 37 °C with shaking at 180 rpm. One millilitre of the overnight culture was used to inoculate 100 mL prewarmed (37 °C) LB broth and incubated in aerobic conditions at 37 °C under shaking at 180 rpm. When bacteria reached the mid-log phase (optical density (OD) at 600 nm = 0.6 ± 0.2), the cells were incubated on ice for 20 min. Bacterial cells were pelleted at 7000× *g* for 10 min, and washed three times: first with 50 mL of ice-cold water, second with 25 mL of ice-cold water, and lastly with 2.5 mL of ice-cold 10% glycerol. Electrocompetent cells were aliquoted and stored at −80 °C.

2.2. Transposon Mutagenesis

Transposon mutagenesis was carried out according to the manufacturer's recommendations (Lucigen). Briefly, 1 µL of EZ-Tn5™ <R6Kγori/KAN-2> Tnp Transposome™ kit (Epicentre Lucigen, bioNova Científica S.L., Madrid, Spain) was mixed with 45 µL of *E. coli* wt electrocompetent cells and included in a 0.2 cm electro-cuvette (Bio-Rad, Madrid, Spain). The cells were electroporated using the Pre-Set protocol from the Gene Pulser Xcell™ Electroporation Systems (Bio-Rad) at 25 µF, 200 Ohms, 2500 V. The electroporated cells were recovered immediately in 1 mL of Super Optimal broth with Catabolite repression (S.O.C.) medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and incubated at 37 °C with shaking (100 rpm) for 60 min. The incubated product was diluted 1:10, and 50 µL aliquots were plated onto LB agar with kanamycin-50 µg/mL. Each colony grown was stored separately at −80 °C in BD Difco™ Skim Milk (Becton Dickinson, Madrid, Spain) for further analysis. The efficiency of the electrocompetent cells was >10^7 colony forming units (CFU)/µg of DNA.

2.3. Biofilm Analysis

Five hundred and thirty-six of a total of 2578 transposon mutants were characterised in terms of biofilm production using a protocol previously standardised in our research group [21]. Briefly,

all isolates were cultured overnight in LB agar at 37 °C in aerobic conditions. The cultures were established by the direct colony suspension method at 37 °C with shaking (180 rpm) in LB broth and then grown overnight. After incubation, colonies were diluted 1:100 in 200 µL of M63 medium (13.5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 5.0 × 10^{-4} g/L $FeSO_4$, 1 mL 1 M $MgSO_4{\cdot}7H_2O$), supplemented with 0.25% glucose and adjusted to pH 7 (with KOH) and tested in 96-well flat-bottomed non-treated polystyrene microtiter plates with lids (Nunc™ Edge 2.0, VWR International, Barcelona, Spain) at 30 °C for 48 h. After incubation, biofilm was quantified using a crystal violet technique previously described by our group [21]. In brief, liquid culture from M63 medium was carefully removed, and the biofilm mass was washed twice with 210 µL of 1× phosphate-buffered saline (PBS) (pH 7.2) and dried at 65 °C for at least 20 min. Biofilms were stained with 200 µL of 2% (*v/v*) solution of crystal violet (CV) and incubated for 10 min at room temperature. Afterwards, the CV was completely removed, washed twice with 1× PBS and heat-fixed at 65 °C for 60 min. The CV was eluted by adding 200 µL of 33% acetic acid. Biofilm formation was measured at 580 nm using a Microplate Spectrophotometer (EPOCH 2 microplate reader, BioTek, Winooski, VT, USA).

The biofilm production of each sample was tested in triplicate, and samples showing an absorbance less than or equal to the positive control (ATCC 25922) were retested. Finally, the mutants were classified into three groups using the paired Student's *t*-test (adjusted $p < 0.05$, considered significant and listed in Supplementary Table S1) comparing the normalised absorbance of each mutant (A Tn) with the absorbance of the reference strain (A ATCC). Only statistically different strains were considered to be LF or high biofilm formers (HF), whereas no statistically significant differences were considered as biofilm formers (F).

2.4. Phenotypic Characterization of Mutants

2.4.1. Growth Curves

To detect deficiencies in bacterial growth, the fitness of the selected mutants was tested. A single colony of each strain was grown overnight in LB broth at 37 °C with shaking at 180 rpm. After incubation, cultures were centrifuged and adjusted at $OD_{600nm} = 1$ with fresh LB, followed by dilution of 1:100 and plated onto 96-well flat-bottomed polystyrene microtiter plates with lids (Nunc™ Edge 2.0, VWR International, Barcelona, Spain). The plates were incubated at 37 °C with double orbital shaking (180 rpm). The absorbance was measured every 15 min for 24 h using the Epoch 2 Microplate Spectrophotometer. Each sample was repeated four times. LB without inoculum was used as a negative control. A plot of the natural log (Ln) of Absorbance versus time during the exponential phase yielded a straight line and the slope of this was equal to the specific growth rate (µ). The µ obtained for each strain was statistically compared to the µ of the wt strain.

Bacterial growth in M63 broth was measured by colony counting instead of Epoch 2, as the low growth of the mutant strain and the colourless medium limits its detection by spectrophotometer. Bacterial viability was also measured using the LIVE/DEAD® BacLight Bacterial Viability Kit, which uses mixtures of SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI). According to the manufacturer, when both dyes are present, it is possible to distinguish between intact and damaged bacterial cells. PI penetrates only into bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain. Thus, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. Therefore, after incubation, biofilms were washed twice with 1× PBS (pH 7.2), cells were detached with a scraper, transferred into an Eppendorf tube, and sonicated at 37 kHz for 2 min using an ultrasonic bath (Fisher Scientific FB 15053, Waltham, MA, USA) following by vigorous vortexing for 1 min. The cells were then stained following the kit protocol and observed in the Olympus IX51 inverted fluorescence microscope. Three biological and technical replicates were done and three fields of each well were observed and imaged using the Fiji ImageJ software. Integrated density (staining intensity), defined as the sum of the values of the pixels in the image selected was measured and the percentage of viability was calculated.

2.4.2. Swimming Assay

A motility assay was performed as previously described [9]. Briefly, transposon mutant bacteria were incubated onto LB-kanamycin plates for 24 h at 37 °C. After incubation, a single colony was streaked in a 0.3% LB agar tube with 0.001% 2,3,5-triphenyl tetrazolium chloride (TTC). The tubes were incubated for 20 h at 37 °C. All completely red-stained tubes were considered positive. A motility assay was performed in triplicate for each strain.

2.4.3. Congo Red Assay

Curli production was determined using the Congo Red (CR) assay as previously described by Prigent-Combaret et al. [20]. YESCA-CR agar plates were made with yeast extract and casamino acid agar (YESCA: 1 g L^{-1} yeast extract, 10 g L^{-1} casamino acids, 20 g L^{-1} agar), and autoclaved at 121 °C. After sterilisation, filter-sterilised CR and Brilliant Blue G (100 µg mL^{-1} and 10 µg mL^{-1} final concentrations, respectively) were added. Transposon mutants were streaked in LB broth and grown at 37 °C overnight. Five microliters of overnight culture were spotted on the centre of the CR agar plate and incubated at 28 °C for 48 h. Dark red colonies were indicative of adhesion fibers while white or light pink colonies were indicative that fibers were not produced.

2.4.4. Hemagglutination Assays

Type 1 fimbriae (pili) can be tested by hemagglutination assays. The *fimH* gene encoded the mannose-specific adhesin, located at the end of the pilus, resulting in hemagglutination in the presence of mannose. Therefore, the presence of hemagglutination in the samples indicated the expression of FimH adhesin. The assay was performed according to the protocol described by Hultgren et al. [22]. Bacterial cells were grown overnight in LB agar. After incubation, each strain was suspended in 1× PBS to obtain an OD_{600nm} of 1.0. One mL of the suspension was centrifuged, and the pellet was resuspended in 0.1 mL of 1× PBS. Twenty-five µL (1×10^9 to 2×10^9 bacteria) were serially diluted in 96-well round-bottomed polystyrene microtiter plates containing 25 µL of 1× PBS in each well. An equal volume of 2.5% previously washed sheep erythrocytes was added and, after mixing, the plates were incubated at 4 °C for 4 to 18 h. The endpoint was defined as the highest dilution at which erythrocyte buttons were not observed. The titre was expressed as the reciprocal of the endpoint and then expressed in Log_2.

2.5. Genotypic Characterization of Mutants

2.5.1. DNA Sequencing

Two mutants with the most significant loss of ability to form biofilm were selected for DNA sequencing. Each strain was cultured onto LB agar plates at 37 °C in aerobic conditions for 24 h. One colony was inoculated in 5 mL of LB broth and placed on ice when the OD reached mid-log phase. DNA extraction was performed using the Bacterial Genomic DNA Isolation Kit (Norgen Biotek Corp, Schmon Pkwy, Thorold, ON, Canada) following the manufacturer's recommendations. Then, DNA was quantified using a Qubit 4 Fluorometer (Thermo Scientific, Waltham, MA, USA).

The genomic DNA material was used for the preparation of TrueSeq Illumina libraries (Illumina Inc, San Diego, CA, USA). The Illumina HiSeq2500 system was used as a sequencing platform with a 2× 125 bp paired-end strategy to generate 6,000,000 reads. The libraries were checked for quality analysis using FASTQC available at (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) implemented by the GPRO suit (https://gpro.biotechvana.com/).

The FASTQ files were pre-processed using Cutadapt software to remove primer adapters [23] and the sequencing quality was established with Prinseq with which reads with less than 50 bp and a concentration of up to 15% of Ns were discarded [24]. Then, de novo genome assemblies were conducted with the SPAdes Genome Assembler [25]. Reads were mapped onto the reference

genome (*Escherichia coli*: GenBank: CP009072.1) using Bwa software [26]. The transposon was detected comparing the reconstructed genome to wt strain using the Mauve software [27].

2.5.2. *purL* Gene Disruption

To confirm the role of *purL* in biofilm formation, the disruption of this gene was done using the λ red recombination system according to the protocol described by Datsenko and Wanner [28]. Briefly, the *E. coli* wt electrocompetent cells were electroporated with 50 ng of a red recombinase expression plasmid (pKD46). Transformants were grown in LB broth with ampicillin 100 μg/mL (Sigma-Aldrich, Milwaukee, WI, USA), and L-arabinose 10 mM (TCI Europe, Zwijndrecht, Belgium), at 30 °C to an OD_{600} of 0.6 ± 0.2 and then were remade as electrocompetent cells.

The deletion of *purL* was obtained by PCR. To generate the PCR fragments, the primers were designed by priming, upstream and downstream, the sites flanking the chloramphenicol resistance gene in pKD3 and with ends homologous to upstream and downstream chromosomal sequences for targeting the *purL* gene. N-terminal deletion primer had a 50-nt 5′extension including the gene initiation codon and the 21-nt sequence 5′-GTGTAGGCTGGAGCTGCTTCG-3′. C-terminal deletion primers consisted of 21 nt for the C-terminal region including the termination codon, 29-nt downstream, and the 20-nt sequence 5′-CATATGAATATCCTCCTTAG-3′. The designed primers were: 5′-CGTTTCCCCCCCTTGGGTACACCGAAAGCTTAGAAGACGAGAGACTTATGGTGTA GGCTGGAGCTGCTTCG-3′ and 5′-CCGGGCTGCAATACCAATGGGTTGACGACTTACCCCAAC TGCTTACGTGCCATATGAATATCCTCCTTAG-3′ (Metabion).

PCR products were gel-purified with the E.Z.N.A. ® Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) and digested with DpnI (Thermo Scientific™, Waltham, MA, USA). 500 ng of the re-purified product were mixed with 100 μL of electrocompetent cells carrying the pKD46 and electroporated. The cells were immediately added to 1 mL S.O.C. medium, incubated at 37 °C with shaking (180 rpm) for 2 h, centrifuged, and resuspended in 500 μL of fresh S.O.C. medium. Then, 250 μL were spread onto each LB agar plate containing chloramphenicol (Cm) 10 μg/mL to select Cm-resistant transformants. Afterwards, the obtained transformants were incubated at 43 °C in LB agar without antibiotics and tested for ampicillin sensitivity to test for loss of the pKD46 helper plasmid. Each colony was stored separately at −80 °C in BD Difco™ Skim Milk (Becton Dickinson, Madrid, Spain) for further analysis. Different PCRs were performed to demonstrate the successful disruption of the *purL* gene. The primers used are listed in Supplementary Table S2.

The growth curve, biofilm formation, and curli fibers formation of the Δ*purL::cat* knockout were also evaluated as previously described.

2.5.3. *purL* Gene Complementation

The knockout strain was complemented to recover biofilm formation capacity. Briefly, purified PCR product of the *purL* gene was ligated to pGEM®-T vector System (Promega, Madison, WI, USA) using the T4 DNA Ligase and cloned into the competent knockout strain. The complemented bacteria were selected in LB agar plates containing ampicillin 100 μg/mL. Each colony was stored separately at −80 °C in Skim Milk for further analysis. Successful complementation was confirmed by PCR using the T7 promoter primer and an internal primer of the *purL* gene (Supplementary Table S2). Then, the biofilm formation, growth curves, and curli production tests of the complemented bacteria were assessed.

2.5.4. Effect of Inosine Addition to the Biofilm Formation Ability of the *purL* Mutant.

To examine the recovery of the ability to form biofilm of the *purL* mutant by the addition of an exogenous source of purine, M63 broth supplemented with double decreasing concentrations of inosine ranging between 50 and 1.56 μg/mL was used for biofilm formation analysis and growth curves, following the protocols previously described. Curli production was also measured by supplementing YESCA-CR agar with inosine 50 μg/mL.

2.6. Proteomic Characterization of Mutants

2.6.1. Protein Isolation

The protein isolation of the wt strain, as well as the proteomic isolation of the Tn263, the *E. coli* mutant selected for proteomic and transcriptional analysis, was performed as follows: the strains were cultured inoculating a single colony into LB broth for 18 h at 37 °C with shaking at 180 rpm to reach a cell concentration of 1×10^9 CFU/mL. Colonies were diluted 1:100 in 70 mL of M63 medium supplemented with 0.25% glucose in a T75 non-treated flask (Thermo Scientific™ Nunc™, Waltham, MA, USA) and incubated at 30 °C for 72 h without shaking. The medium was carefully replaced by a fresh medium every 24 h. Subsequently, the medium was aspirated to remove planktonic cells, and flasks were washed twice with 20 mL of 1× PBS (pH 7.2) to remove unattached or loosely attached cells. Cells were then resuspended with 25 mL of 10 mM Tris-KCl (pH 7.8, 150 mM KCl) and sonicated at 37 kHz for 4 min using an ultrasonic bath (Fisher Scientific FB 15053, Waltham, MA, USA). Cells were harvested in 50 mL sterile falcon tubes (Deltalab, Rubí, Spain) and spun down at 3500× *g* for 10 min at 4 °C. The cell pellet was washed 3 times in 50mL of Tris-KCl and finally transferred into an Eppendorf tube and pelleted at 12,000× *g* for 10 min at 4 °C. The pellet was homogenized in lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 2% ASB-14), and supplemented with protease inhibitor (GE Healthcare, Chicago, IL, USA). Bacterial cells were broken by sonication for 10 min with a cycle of 20 s on/59 s off at 4 °C on ice-water. After cell disruption, the samples were centrifuged at 3500× *g* for 15 min at 4 °C, and the supernatant was aliquoted and stored at −80 °C. The protein extracts were quantified using the 2-D Quant Kit and purified with the 2-D Clean-Up Kit (GE Healthcare). Fifty µg of protein was added to 0.5% of immobilized pH gradient buffer 4–7 and 20 mM of dithiothreitol (DTT). Each sample was assessed in three biological replicates.

2.6.2. Two-Dimensional SDS-PAGE Analysis

Two-dimensional sodium dodecyl sulfate polyacrylamide gel (2D SDS-PAGE) was performed as previously described [29]. Briefly, the Immobiline DryStrip Gels (IPG strips) (GE Healthcare) were hydrated following the manufacturer's recommendations for 18 h at room temperature. The protein sample was loaded onto an IPG strip for first dimension separation by isoelectric focusing using 24 cm pH 4–7 linear IPG strips for 20 h (200 V for 2 h, 500 V for 3 h, 1000 V for 4 h, and 8000 V for 11 h, 20 °C) in an IPGphor I (GE Healthcare). After focusing, each strip was equilibrated in 10 mL of equilibration buffer (6M Urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS and 0.002% bromophenol blue supplemented with DTT or iodoacetamide and 1% DTT) for 15 min and acetylated with iodoacetamide, and subsequently placed onto 12.5% polyacrylamide gels (24 cm × 20 cm) prepared as described by Laemmli [30]. The second dimension was performed using an Ettan™ DALT six system (GE Healthcare). Gels were run at 20 °C using 80 V, 10 mA/strip, and 1 W/strip for 1 h, followed by 500 V, 40 mA/strip and13 W/strip until the bromophenol blue tracking front had run off the end of the gel.

2.6.3. Gel Staining and Protein Detection

Two-dimensional gels were silver stained as described previously [31]. Briefly, the gels were fixed for 18 h in 40% ethanol and 10% acetic acid, followed by incubation in sensitizing solution for 1 h in 30% ethanol with 0.02% (*w/v*) sodium thiosulfate. The gels were washed three times in distilled water for 5 min and treated with 0.1% (*w/v*) silver nitrate for 30 min. The gels were washed again twice with distilled water for 1 min. The developing step was performed with 3% (*w/v*) sodium carbonate and 0.025% (*v/v*) formaldehyde until the desired contrast was reached. Developing reaction was stopped with 1.5% (*w/v*) EDTA (Ethylenediaminetetraacetic acid)-Na_2 for 45 min. Finally, the gels were washed twice with distilled water.

2.6.4. Mass-Spectrometry Analysis for Orbitrap

Selected protein spots were excised from the gels digested with trypsin and analysed by liquid chromatography coupled to mass spectrometry (Orbitrap Velos, Thermo, Waltham, MA, USA), as described previously [32]. Proteins were identified through searching against *E. coli* proteins found in the UniProt database, using Mascot (Matrix Science, London, UK) to search the SwissProt database (2018_11, taxonomy restricted to *E. coli* proteins). Tandem mass spectrometry (MS/MS) spectra were sought with a precursor mass tolerance of 10 ppm, fragment tolerance of 0.05 Da, trypsin specificity with a maximum of two missed cleavages, cysteine carb-amido-methylation set as fixed modification, and methionine oxidation as variable modification. The significance threshold for the identifications was set at $p < 0.05$, with a minimum ions score of 20.

2.6.5. RNA Isolation and cDNA Synthesis

E. coli ATCC 25922, the transposon mutant strain Tn263, as well as three biofilm-forming and three non-biofilm-forming clinical isolates were used for further gene expression analyses. Clinical strains belonging to a collection of our research group were collected over six months between 2016 and 2017 from different hospitals in Barcelona, Spain). All strains had been previously identified by MALDI-TOF mass spectrometry and their antimicrobial profile was tested according to the M100 guidelines (Clinical & Laboratory Standards Institute, CLSI 2019) [33]. Origins of isolation and antibiotic resistance profiles are shown in Supplementary Table S3. The strains were cultured by inoculating a single colony into LB broth for 18 h at 37 °C with shaking at 180 rpm to reach a cell concentration of 1×10^9 CFU/mL. Colonies were diluted 1:100 in 70 mL of M63 medium supplemented with 0.25% glucose in a T75 flask for 72 h and 30 °C without shaking. Subsequently, the medium was aspirated to remove planktonic cells, and flasks were washed twice with 20 mL of sterilized 1× PBS (pH 7.2). Cells were resuspended in 3 mL of 1× PBS and treated with 6 mL of RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) and vortexed and centrifuged at 5000 rpm for 20 min at room temperature. The supernatants were discarded, and the pellets were treated with TE-lysozyme buffer (3 mg/mL in TE buffer pH 7.4). Afterwards, RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The total RNA was eluted in 50 μL of RNase-free water. RNA was isolated from four independent samples.

The extracted RNA was treated with DNA-free™ Kit DNase Treatment and Removal Reagents (Ambion, by Life Technologies AM1906, Waltham, MA, USA) to remove DNA contamination. After DNase treatment, RNA was tested for residual genomic DNA contamination by polymerase chain reaction (PCR) amplification of the *adk* gene and gel electrophoresis.

The total RNA concentration was quantified using NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). An A260/280 ratio between 1.80 and 2.10 was considered optimal for RNA quality. cDNA was synthesized using 500 ng of RNA in a reaction volume of 20 μL and the qScript cDNA SuperMix reagent (Quanta Bioscience, 95048-100, Gaithersburg, MD, USA). The PCR amplification protocol consists of one incubation step at 25 °C for 5 min, a DNA polymerization step at 42 °C for 30 min, and a final enzyme deactivation step at 85 °C for 5 min. The final PCR product was diluted 1:10 and stored at −20 °C. cDNA was used as a template for quantitative reverse transcription PCR (qRT–PCR) assays.

2.6.6. Gene Expression by qRT-PCR

qRT-PCR was carried out using 10 μL of PowerUp™ SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, A25742, Foster City, CA, USA), 1 μL of primer mix (each reverse and forward primers at a final concentration of 0.5 μM), 4μL of RNase-free water and 5μL of cDNA template.

Reactions were conducted in duplicate and the *arcA* gene was used as the reference gene for normalisation of CT-values [34,35]. A non-template control reaction mixture was included for each gene. Amplification was performed using StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) being the cycling conditions: a pre-incubation step of 95 °C at 15 min, followed

by an amplification step of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s repeated for 44 cycles, and a melting curve analysis from 60 °C to 95 °C in 0.3 °C intervals. The relative fold-change of mRNA transcripts in biofilms compared to mutant cultures was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Supplementary Table S4. The primers designed in this study were made using Primer Express Software v.3.0.1 (Applied Biosystems). Data analysis was based on at least three independent experiments.

2.7. Data Plotting and Statistical Analysis

All statistical analyses were performed using GraphPad Prism v8.0.2 software (La Jolla, CA, USA) unless otherwise stated. Graphs were created using GraphPad Prism v8.0.2 software and Tableau Software (Seattle, WA, USA). Growth rates were evaluated via two-tailed Student *t*-test. One-way ANOVA followed by post hoc Dunnett's multiple comparison tests were used to analyse colony counting in M63, LIVE/DEAD assay, and biofilm quantification after the addition of inosine. Statistical quantification of relative protein levels was analysed with Progenesis SameSpots 4.6.206 (Totallab, Newcastle, UK) and evaluated via ANOVA. The relative transcript levels of selected genes in wt strain, Tn263 mutant and clinical isolates were calculated using the $2^{-\Delta\Delta Ct}$ method. Two-tailed student *t*-test was performed to compare wt vs Tn263 and biofilm-former vs non-biofilm former Differences were considered statistically significant with a p value < 0.05.

3. Results

3.1. Mutant Selection by Biofilm Assays

A total of 2578 *E. coli* mutants by transposon insertion were generated, of which 536 were analysed in this study. The mutants were further studied for their ability to form biofilm and compared to the wt strain. Mutants presenting reduced biofilm-formation capacity (Table S1) were selected. A total of 116 mutants showed an altered biofilm formation rate in comparison with the wt strain. Among these, 20 were classified into the low biofilm formers (LF) group (3.73%) with an absorbance between 0.065 and 0.584, 420 mutants classified as formers (F) (78.36%) with an absorbance from 0.588 to 1.414 and finally 96 were classified into the high biofilm formers (HF) (17.91%) with an absorbance higher than 1.414 (Figure 1). Into the low biofilm formers, Tn263 and Tn463 mutants showed the most significant loss of biofilm formation with values of absorbance of 0.065 and 0.159, respectively.

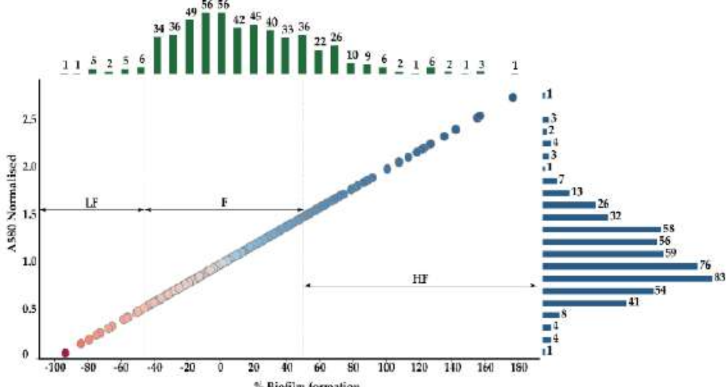

Figure 1. Scatter plot and marginal histograms of the mutants. Upper bars (green) indicate the number of strains in the respective percentage of biofilm formation. Right bars (blue) indicate the number of stains in the respective absorbance values. Each spot represents a mutant where red colour indicates low biofilm formation rates and blue corresponds to high biofilm formation rates. LF: low biofilm former; F: biofilm former; HF: high biofilm former; A: absorbance.

3.2. Phenotypic Characterization of Mutants

The 20 mutants classified into the low biofilm-forming capacity group (LF) were assessed with a phenotypic comparative study.

3.2.1. Growth Curves in LB Broth

Transposon insertion can affect essential metabolic pathways and alter normal physiological development. To detect these possible defects, growth curves were evaluated on 20 LF mutant strains and compared to the wt strain (Figure 2). The analysis of the specific growth rate (μ) performed in LB broth showed statistically significant differences in the Tn463 mutant in comparison to the wt ($p < 0.0001$). The specific growth rates (μ) of the other mutant strains were similar to the wt, and no statistically significant differences were found ($p > 0.05$). Figure 2 shows the entire growth curve measuring absorbance every 15 min until 24 h of incubation.

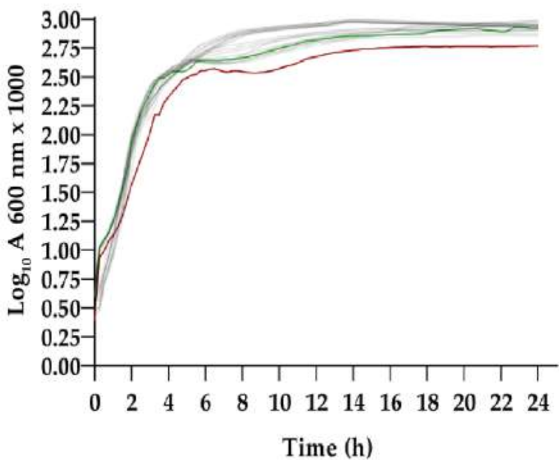

Figure 2. Fitness assay in Luria Bertani (LB) broth. Green represents the wild type curve; Red represents Tn463. Grey represents the other 19 low biofilm-former (LF) transposon mutants, including Tn263. Growth rates were statistically evaluated via two-tailed Student *t*-test.

3.2.2. Swimming, Congo Red and Hemagglutination Assays

In this section, 20 mutants were examined according to the phenotypic characterization of non-biofilm-forming mutants. The results of the swimming, curli fibers production and hemagglutination assays are summarized in Table 1.

In the swimming assay, no samples were found to be non-motile, indicating that defects in biofilm formation are not related to defective flagella. Supplementary Figure S1 shows an example of swimming classification using a defective mutant in the *flhD* gene as non-motile control and wt as motile control. The ability to form curli fimbriae was evaluated using the CR assay in YESCA-CR plates. In this assay, the Tn463 and Tn263 mutants showed significant differences caused by altered or defective synthesis of curli. The results obtained in this assay are further discussed in Section 3.3.2. Finally, all selected mutants showed positive hemagglutination, suggesting that type 1 pili are not involved in the lack of biofilm formation in these mutants. Since Tn463 and Tn263 showed the the most significant loss of biofilm formation and some changes in these assays, we analysed only these mutants in the next section.

Table 1. Phenotypical results of selected mutants.

Sample	Swimming	Curli Production	Hemagglutination (Log_2)
Tn5	+	+	10
Tn29	+	+	10
Tn42	+	+	10
Tn82	+	+	10
Tn90	+	+	10
Tn119	+	+	10
Tn143	+	+	10
Tn 249	+	+	10
Tn 251	+	+	10
Tn 262	+	+	10
Tn263	+	-	10
Tn 337	+	+	10
Tn 373	+	+	10
Tn 406	+	+	10
Tn 420	+	+	10
Tn 425	+	+	10
Tn 457	+	+	10
Tn 463	+	-	10
Tn 467	+	+	10
Tn 474	+	+	10

+: the mutant strain presents this characteristic; -: the mutant strain does not present this characteristic.

3.3. Genotypic Characterization of Mutants

3.3.1. DNA Sequencing

Since this study focused on low-forming strains after transposon insertion, the Tn263 and Tn463 mutants were selected for DNA sequencing. Although this was not within the scope of the present study, the characterisation of the mutants with increased biofilm production could also lead to interesting results and warrants further investigations.

According to the analysis of transposon insertion realised in collaboration with BIOTECHVANA (Valencia, Spain), in the Tn463 mutant, the transposon was inserted in the *csgA* and *cysB* genes. The *csgBAC* operon encodes the curli major subunit protein CsgA, the curli nucleator protein CsgB, and the periplasmic chaperone CsgC [36]. The importance of curli fibers in biofilm formation has previously been demonstrated as being linked to the synthesis of the exopolysaccharide cellulose, a complementary factor of the biofilm matrix [37]. *cysB* gene encodes for CysB protein which is a positive regulator of gene expression for the cysteine regulon, a system of ten or more loci involved in the biosynthesis of L-cysteine from inorganic sulfate. Since *csgA* is a gene that has been previously related to biofilm formation, we focused only on Tn263 for further analysis. In Tn263, the transposon was only inserted in the *purL* gene. *purL* encodes for phosphoribosyl-formyl-glycinamidine synthase, involved in the de novo purine biosynthesis pathway using 5-phospho-α-d-ribose 1-diphosphate (PRPP) as a precursor for producing inosine monophosphate (IMP), which is the precursor of adenosine monophosphate (AMP) and guanine monophosphate (GMP) (Figure 3). Interestingly, in our mutant, the transposon insertion is located after 134 bp, position 45 in the protein affecting the N-terminal domain and producing an unstable and non-functional protein but not affecting the gene expression. Therefore, purine synthesis appears as an essential function whereby bacteria produce nucleotides for the synthesis of DNA and RNA. Given the impact on the purine biosynthesis pathway, other metabolism alterations in Tn263 were investigated using 2D SDS-PAGE analysis and subsequent protein identification with orbitrap.

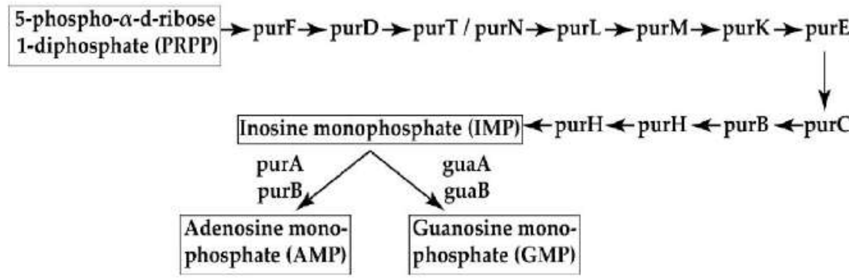

Figure 3. De novo purine biosynthesis in *E. coli*. Adapted from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps.

3.3.2. Confirmation of the Role of the *purL* Gene in Biofilm Formation Using Its Knockout Strain

The Δ*purL::cat* (Cm^R), Tn263, and wt strains were cultivated in M63 broth. The mean value of colony-forming units showed statistically significant differences in biofilm as well as planktonic culture in both Δ*purL::cat* and the Tn263 mutant strains compared to the wt strain after 72 h of incubation in M63 broth. In the same line, the measure of integrated density showed significant differences in the total number of cells in biofilm ($p = 0.0002$ and $p = 0.0008$, Tn263 and *purL* mutant, respectively). Nonetheless, the percentages of live and dead bacteria were highly similar between them (Table 2).

Table 2. Colony-forming units and Live/Dead BacLight Bacterial Viability assay.

	Log_{10} CFU/mL ± SD		Live/Dead		
	Biofilm	**Planktonic**	**Integrated Density**	**Live (% ± SD)**	**Dead (% ± SD)**
wild type	9.09 ± 0.24	8.99 ± 0.05	$2.03 \times 10^7 \pm 4.86 \times 10^6$	85.40 ± 6.30	14.60 ± 6.30
Tn263	6.40 ± 0.51	6.96 ± 0.01	$9.69 \times 10^6 \pm 2.79 \times 10^6$	86.32 ± 4.01	13.68 ± 4.01
Δ*purL::cat*	6.22 ± 0.44	7.02 ± 0.02	$1.11 \times 10^7 \pm 2.34 \times 10^6$	80.38 ± 6.88	19.62 ± 6.88

However, similar growth curves of the Δ*purL::cat* and the Tn263 mutant strains compared to the wt strain were observed when the M63 culture media was supplemented with inosine (50 µg/mL). The complemented strain Δ*purL*/*purL*+ showed similar growth curves to the wt strain in LB as well as in M63. Supplementation with inosine did not have a significant effect on the growth curve of the complemented strain. It should be noted that the general growth in the supplemented M63 broth shows differences in the Log phase compared to the Log phase obtained with LB broth (Figure 4).

As already observed with the Tn263, the Δ*purL::cat* mutant was not able to form biofilm in M63 broth. However, when the medium was supplemented with inosine (50 µg/mL), both the Δ*purL::cat* and the Tn263 mutants recovered the ability to form biofilm. Likewise, no statistically significant differences after One-way ANOVAs followed by post hoc Dunnett's multiple comparisons tests were found using inosine concentrations ranging between 25 and 50 µg/mL for the Δ*purL::cat* and the Tn263 mutants compared to the wt ($p > 0.05$). Nevertheless, inosine concentrations below 25 µg/mL were insufficient to recover the ability to form a biofilm by the *purL* affected strains (Figure 5). Then, after complementation with the *purL* gene using the pGEM®-T vector, the knockout strain also recovered the ability to form biofilm, and the addition of inosine did not have a significant effect on its biofilm formation. The complemented strain Δ*purL*/*purL*+ shows similar absorbance values than the wt strain. The addition of inosine does not have a significant effect on its ability to form biofilm.

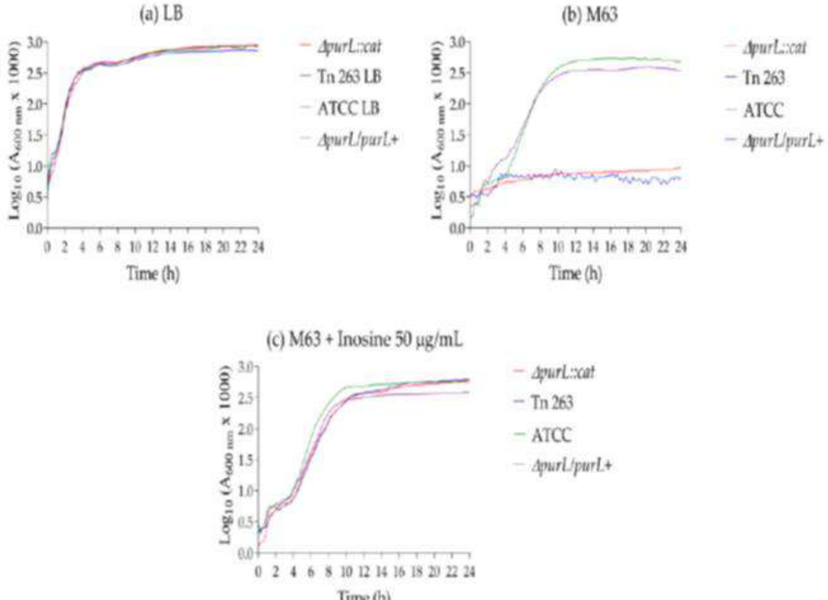

Figure 4. Fitness assay. (**a**) Growth curves in LB broth (**b**) Growth curves in M63 broth. (**c**) Growth curves in M63 broth supplemented with inosine (50 μg/mL). Growth rates were statistically evaluated via two-tailed Student *t*-test.

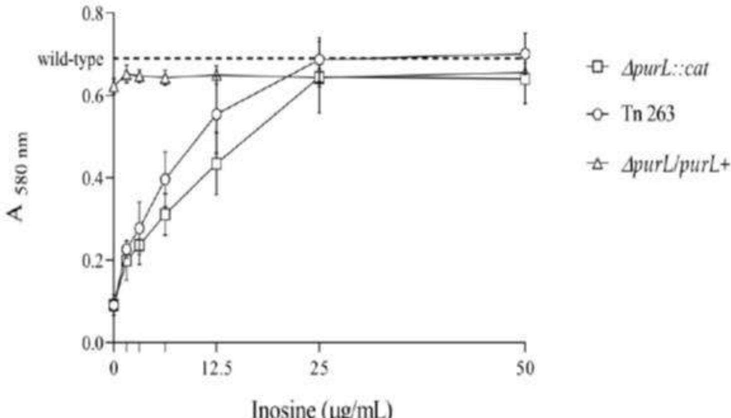

Figure 5. Biofilm formation of Δ*purL::cat* mutant, Tn263, and the complemented strain Δ*purL/purL+* using different concentrations of inosine. Each point in the curve represents mean values of A_{580nm} after 48 h of incubation; vertical bars correspond to standard deviations. The dashed line shows the absorbance value of the wild type strain, used as control. The results were analysed by One-way ANOVAs followed by post hoc Dunnett's multiple comparisons tests.

Curli production was also measured by CR assay. Δ*purL::cat* and Tn263 mutants showed an inability to bind CR and produced less curli fibers compared to the wt and the complemented strains. Curli production was restored in the mutants by supplementing agar medium with inosine 50 μg/mL, demonstrating the interplay between curli production, purine biosynthesis pathway, and biofilm formation (Figure 6).

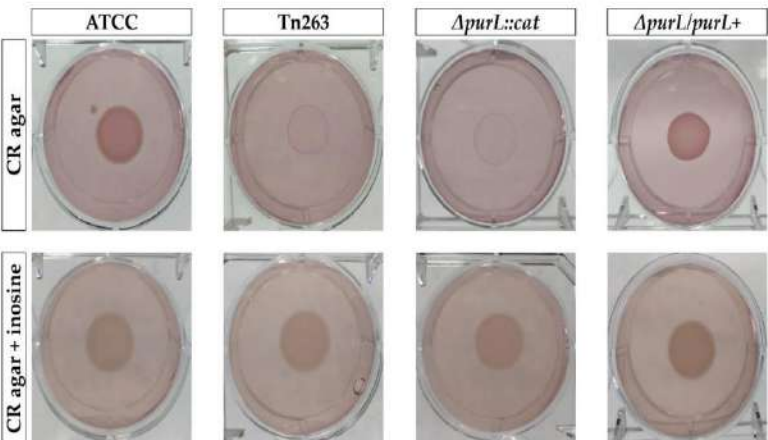

Figure 6. Curli production. Dark red colony in yeast and casamino acid agar (YESCA-CR AGAR) (ATCC and Δ*purL/purL+*) represents a curli producer, and light pink colonies (Tn263 and Δ*purL::cat*) were associated with the defective phenotype. The defective phenotype of both mutants was restored in the YESCA-CR + Inosine agar.

3.4. *Proteomic Characterization of Mutant Tn 263*

3.4.1. Comparative Proteomic Analyses of the wt and Tn263 by Two-Dimensional SDS-PAGE

To investigate the effect of transposon insertion at a molecular level, the Tn263 mutant was compared to the wt strain using two-dimensional SDS-PAGE and mass spectrometry. Three biological replicates of each sample were studied (Figure 7, Supplementary Figure S2, and Supplementary Figure S3). Thirteen statistically significant spots were identified, 12 present in the wt strain, and one in the mutant strain. Among these, only spots with a score over 25 were considered for posterior analyses (ANOVA $p < 0.05$) (Supplementary Table S5). The molecular weight (Mw) and isoelectric point (pI) of proteins were determined using MASCOT software and are in line with their theoretical Mw/pI. The higher Mw might be the result of covalent binding, although lower Mw may be produced by proteolysis or post-translational changes. Figure 7 provides a representative image of 2D-gels in which spot differences are highlighted with arrows.

After mass-spectrometry identification and following pathway analysis, 13 proteins were classified into three categories: metabolism and cell maintenance; genetic information processing and signalling and cellular processes.

Within the metabolism regulation six proteins were included: CysJ, IlvC, ElbB, MgtA, Pta and AdhE. CysJ is a nicotinamide adenine dinucleotide phosphate (NADPH) flavin oxidoreductase that participates in the sulfite reductase complex (CysJ8I4). This protein is encoded by the *cysJ* gene, which plays an essential role in sulphur metabolism, required for de novo biosynthesis of L-cysteine. Aside from biosynthesis pathways, *cysJ* is also related to the detoxification system of toxic and mutagenic N-hydroxylated nucleobases [38]. In addition, IlvC, a Ketol-acid reducto-isomerase encoded by the *ilvC* gene, is seen to participate during valine, leucine, and isoleucine biosynthesis. Glyoxalases are principally involved in the conversion of glyoxal to glycolate, therefore facilitating the conversion of toxins to a non-hazardous product [39]. *E. coli* possesses four glyoxalases which belong to the DJ-1 superfamily, i.e., HchA, YajL, YhbO, and ElbB, the latter present in our analysis. On the other hand, MgtA, a magnesium-transporting adenosine triphosphatase (ATPase), has a crucial function in transmembrane transport of electrons or protons. Phosphate acetyltransferase encoded by the *pta* gene is the first enzyme of the acetate pathway in the aerobic metabolism. The enzyme catalyzes the interconversion of acetyl-phosphate and acetyl coenzyme A. Finally, the only protein overexpressed in

the Tn263 mutant was an aldehyde-alcohol dehydrogenase, encoded by the *adhE* gene ($p = 0.0025$). Under anaerobic conditions, AdhE catalyzes the reduction of acetyl-CoA to acetaldehyde, leading to an increase of NAD^+.

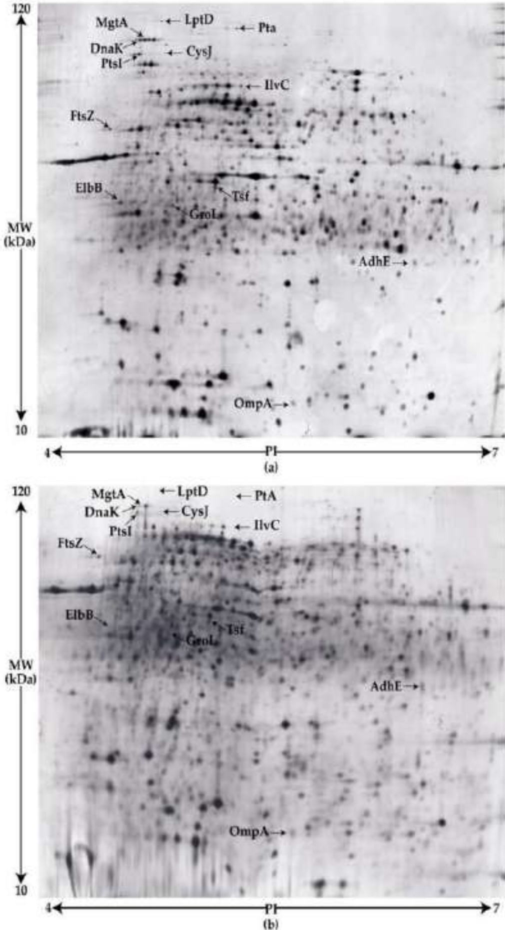

Figure 7. Two-dimensional sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) images. (**a**) wt strain; (**b**) Tn263. The arrows mark significant differences (ANOVA $p < 0.05$) among the strains detected by Progenesis SameSpots 4.6.206. Further information about each spot identified can be found in the supporting information (Supplementary Table S5).

Regarding the proteins identified within the genetic information processing group, we identified the elongation factor thermostable (EF-ts) which is associated with the elongation factor Thermo-unstable (EF-Tu) and regulates the exchange of Guanosine 5′-Diphosphate (GDP) to GTP via aminoacyl-tRNA. The union of EF-ts to ternary structures (EF-Tu-GTP-aa-tRNA) could increase the maximum rate of translation [40]. The cell division protein FtsZ regulated by GTP hydrolysis was also detected. This protein marks the site where the Z-ring structure divides the cell. Z-ring mutation could alter the structure and lead to both the development of functional and non-functional cells [41]. Included in the list there was also the DnaK chaperone, the dominant bacterial heat shock protein (Hsp 70). This is involved in protein secretion and chromosomal DNA replication and interacts with σ^{32} for controlling heat shock response. The last protein was the 60 kDa chaperonin GroL. The GroL chaperonin, also stimulated by σ^{32}, mediates the assembly of unfolded polypeptides generated under

stress conditions, and together with its regulator GroES, it is necessary for the proper folding of specific proteins.

Last, within the signalling and cellular process, we found the LptD (lipopolysaccharide transport) protein which, in combination with LptE protein, has an essential role in the production of lipopolysaccharide (LPS), the predominant component at the surface of the outer membrane (OM) of all Gram-negative bacteria. We also found the phosphoenolpyruvate-protein phosphotransferase enzyme 1 (PtsI) which participates in the phosphotransferase system related to phosphorylation of carbohydrates in *E. coli*. Finally, outer membrane protein A (OmpA) was identified as an abundant protein in *E. coli*, and it plays a significant role in maintaining cell integrity [42].

3.4.2. Verification of Biofilm-Related Genes by q-RT-PCR

The protein coding-genes corresponding to the proteins previously identified by SDS-PAGE and mass spectrometry were analysed to investigate possible translational alterations in the mutant sample. Further validation of these interesting targets was conducted by qRT-PCR between Tn263, the wt strain and the three biofilm-formers and three non-biofilm-formers clinical isolates.

mRNA detection by qRT-PCR and the subsequent analysis by $2^{-\Delta\Delta Ct}$ method showed that three genes were downregulated (*dnaK*, *groL*, and *adhE*) and six genes were overexpressed (*lptD*, *cysJ*, *pta*, *ilvC*, *elbB* and *ptsI*) in the Tn263 mutant compared to the wt strain. Four genes did not present changes among groups (*tsf*, *ftsZ*, *ompA*, and *purL*) (Figure 8a). To investigate whether these expression changes were related to the decreased biofilm formation capacity of the mutant, we assessed the expression levels of these genes in a collection of well-characterized *E. coli* clinical isolates. Two genes, *adhE* and *ptsI* presented a significant upregulation in the non-biofilm producers compared to the biofilm producer strains ($p = 0.0347$ and $p = 0.0169$, respectively) (Figure 8b). In the first case, the Tn263 mutant showed upregulation of the AdhE protein, while it showed a low mRNA expression. However, mRNA expression in non-biofilm-formers remained upregulated. In the last case, the production of the PtsI protein was detected as being upregulated in the wt strain, whereas mRNA transcriptional levels were upregulated in both, the Tn263 and non-biofilm-formers.

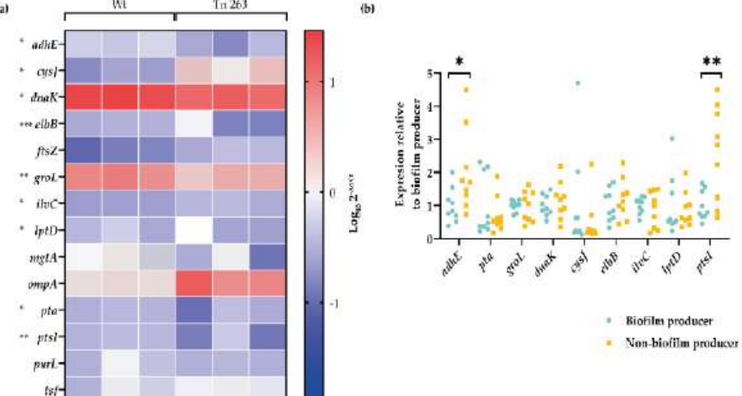

Figure 8. Analysis of the expression of selected genes in the wt and Tn263 mutants and in clinical isolates. (**a**) Heatmap showing the expression levels of biofilm-related genes in the wt and Tn263 mutant strains. Expression is presented as a logarithm of their $2^{-\Delta\Delta Ct}$. Each square shows a biological replicate (n = 3 vs 3). (**b**) Scatter plot showing the expression of selected genes in biofilm producer and non-biofilm producer clinical isolates. Expression is normalised relative to the average expression of the biofilm producer isolates per each gene (n = X vs X). Each clinical isolate was performed in triplicate. T test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

Bacteria have largely been investigated as planktonic cells. However, in many cases, bacteria grow in communities known as biofilms. These structures offer evolutionary advantages over their competitors and enable them to overcome environmental stresses. It is therefore important to understand the factors involved in the development of microbial biofilms that give them phenotypes different from their planktonic counterparts.

"Omics" techniques, such as high-throughput DNA sequencing, transcriptomic approaches, or proteomics, can clarify the underlying mechanisms of biofilm production. Progress in this field has indeed led to numerous studies that provide insight into molecular approaches [43].

The present study aimed to determine new genes involved in biofilm formation in *E. coli* using phenotypic and molecular tools comparing *E. coli* wt strain and the isogenic defective-biofilm mutants (Tn) generated by transposon insertion. Although the characterisation of the mutants with increased biofilm production was not within the scope of the present study, this could also lead to interesting results and warrants further investigations.

In the transposon mutant library, we found deficient mutants in biofilm formation. Two of these with the most significant loss of biofilm formation capacity were selected for the following analyses.

Tn463 was initially selected as a non-biofilm-forming mutant in the first phenotypic assays. However, it was later discarded because it had altered fitness and expression in curli fimbriae compared to the wt strain. Besides, the transposon was inserted into the *csgA* gene, a previously described gene that is involved in biofilm formation.

The second one, Tn263 mutant, differed from the wt in its proteomic and transcriptional profiles. DNA sequencing revealed a transposon insertion in the *purL* gene, which is involved in the de novo purine biosynthesis pathway. This pathway plays a critical role in the synthesis of DNA and RNA. In *E. coli*, the structural gene *pur* has various loci either individually (*purT, purL, purC, purA*) or collectively within operons (*purF, purHD, purMN, purEK*) [44]. Concretely, *purL gene* codes for formyl-glycinamide ribonucleotide amido-transferase (FGAR-AT), which is an enzyme that catalyses the fourth step of the purine biosynthetic pathway. This is an ATP-dependent conversion of formyl-glycinamide ribonucleotide (FGAR) and glutamine to yield N-formylglycinamidine ribonucleotide (FGAM), adenosine diphosphate (ADP), Pi, and glutamate. In our mutant, the transposon was located in the N-terminal domain of the *purL* gene. Previous studies related three principal domains in *purL:* the glutaminase, the FGAM synthetase, and the N-terminal domains [45]. The glutaminase domain produces ammonia, which is transported to the FGAM synthetase domain. The N-terminal domain possesses an essential function for ammonia channel formation and posterior union between the two catalytic domains [46]. Changes in the N-terminal domain could potentially alter the interactions between other structures and block their function without transcriptional modification.

In order to determine whether the *purL* mutation could cause a fitness deficiency that would impede the biofilm formation, the mutant strain was grown in LB, without changing its growth rates, which suggests that the *purL* mutation does not affect its fitness in this growth medium. However, fitness was altered in M63 due to the alteration in the purine biosynthetic pathway. Biofilm formation was carried out in M63 medium at 30 °C to favour the expression of genes involved in the biofilm formation [20,21,47–50] as curli fimbriae, aggregated amyloid structures which promote cell aggregation and attachment to abiotic surfaces. They are promoted at a temperature less than 32 °C inducing transcription of the *csgDEFG* operon [51]. Nonetheless, Tn263 and *ΔpurL::cat* mutants showed poor biofilm formation in the M63 medium compared to the wt strain. On the other hand, when we used the M63 broth supplemented with inosine in different concentrations, both mutants, Tn263 and *ΔpurL::cat*, regained the ability to form biofilm. Since both strains can form biofilm in the inosine-supplemented M63 medium, the lack of biofilm formation could be attributed to the *purL* disruption. The result was confirmed by the complementation of the knockout strain. Thus the lack of purines tends to reduce RNA synthesis by RNA polymerase or amino acid starvation resulting in a deficient biofilm phenotype. Nhu et al. found that the de novo purine biosynthesis is a critical pathway in curli production [36].

They hypothesise that a disruption in this pathway reduces the cyclic-di-GMP concentration inside the cell triggering the transcription of the master regulator *csgD*. The authors found that *purF, purD, purM* and *purK* mutants were unable to bind CR on YESCA-CR agar. In the same line, Garavaglia et al. also found that the mutation of the purine biosynthetic gene *purH* resulted in the inability to produce curli fibers [52].

Our results support these previous findings because the Δ*purL::cat* mutant exhibits a curli-deficient phenotype, resulting in decreased biofilm. However, after supplementation of the growth medium with inosine, both mutants, Tn263 and Δ*purL::cat*, restored curli assembly suggesting that *purL* alteration and subsequent dysfunction in the purine pathway hinder the efficient transcription of the *csgDEFG* operon and *csgD* expression [52].

Biofilms are an important virulence factor involved in surface colonisation and subsequent infection, often causing chronic infections. A change in the purine synthesis pathway could reduce the colonisation rate and thus decrease biofilm infection. For example, disruption in the *cvpA-purF* or only the *purF* genes related to the de novo purine biosynthesis reduces uropathogenic *E. coli* (UPEC) internalization into bladder epithelial cells without alteration in epithelium colonisation [53]. Along the same lines, one study demonstrated that *Burkholderia*, defected in *purl* was unable to establish symbiotic accommodation in *Riptortus* sp. *Burkholderia* did not colonize *Riptortus* sp, even though the bacteria were capable of colonizing the host midgut. These results suggested that purine biosynthesis also could play an essential role in infection.

After finding that transposon was inserted into the *purL* gene, additional studies were conducted on the changes that this insertion caused at proteomic and transcriptional levels between wt and Tn263.

Comparative proteome analysis revealed 13 proteins statistically significant among wt and Tn263 (Figure 7). Among these proteins, five were found to be closely associated with biofilm regulation. The two chaperones, DnaK and GroL, are involved in stress response that usually results in the passage from individual cells to biofilm, inducing the loss of the flagella and performing maintenance activity in mature biofilm. Specifically, DnaK was involved in the transcriptional regulation of *flhDC*, responsible for the flagellum biogenesis, an important organelle associated with biofilm formation, adhesion, and colonisation [54]. Moreover, a recent work by Sugimoto et al. [55] showed that the DnaK protein contributes to curli formation. Via *rpoS*, DnaK modulates the expression of the *csgDEF* operon which encodes for CsgD (master transcriptional regulator of curli) acting as a positive regulator of the *csgBAC* operon. This last operon encodes the CsgA and CsgB units, the major and minor structural components, respectively. On the other hand, studies in *Cutibacterium acnes* hypothesized that the GroL protein might contribute to the organisation of the biofilm matrix given its ability to bind to external DNA [56]. For this reason, the detection of proteins involved in maintaining a mature biofilm maintenance in the wt strain supports the idea that the Tn263 mutant was unable to form a biofilm.

Pta enzyme could be indirectly modulate the biofilm formation through acetate intermediates that can activate regulatory cascades of biofilm formation [57]. For example, acetylation produced by acetyl-coA from CpxA and UvrY, both members of the two-component regulatory system or RcsB, are involved in numerous regulatory DNA regions such as colanic acid synthesis, which enhances the 3D structure of the biofilm [58–60].

Another protein involved in biofilm formation that was detected in wt strains was PtsI. In this case, previous studies on *Bacillus cereus* showed that mutations in the *ptsI* gene cause a 70% decrease in biofilm rates [61]. In avian pathogenic *E. coli* (APEC), *ptsI* deletion resulted in motility and biofilm-deficient bacteria, among other pathogenesis and phenotypic changes [62]. Furthermore, in *E. coli*, the internalisation of AI-2, a QS regulator, via Lsr activation, is mediated through *ptsI* phosphorylation [63]. Our results reinforce the hypothesis that PtsI is synthesized when bacteria develop the biofilm, given that PtsI is used to internalise AI-2 in the matrix. The fact that the mutant strain synthesises a lower amount of PtsI indicates that it is not in confluence and, therefore, it does not need to synthesize the protein. The last protein detected at high rates and associated with biofilm

regulation in wt strain was OmpA. Indeed, the study by González Barrios et al. showed that the presence of an isogenic OmpA mutant decreases biofilm formation [64].

Finally, the only protein with high rates in Tn263 was AdhE. We hypothesise that the mutation in the *purL* gene leads to a hypoxic state, therefore activating anaerobic respiration. This idea is reinforced by studies carried out by Leonardo et al. who showed that using glucose as a carbon source in anaerobic conditions decreases NADH/NAD$^+$ ratio and changes the expression of the *adhE* gene [65]. Hence, the *adhE* gene would be regulated at the transcriptional level by the NADH/NAD$^+$ ratio.

Colón-González et al. also explained that anaerobic growth does not support biofilm formation in *Escherichia coli* K-12 by the enzymatic activity of the AdhE protein. *adhE* gene is induced during oxygen deficiency, and the authors found significant differences of growth in Congo red agar, where aerobically grown cells were red (curli +) and anaerobically grown cells were white (curli −) [66]. This suggests a possible interaction between AdhE expression and defective curli fibers under anaerobic conditions. AdhE was found to be upregulated in Tn263 compared to the wt strain and does not produce curli fibers or biofilm. However, this gene is not upregulated among non-biofilm forming clinical strains. Therefore, we hypothesise that the expression of AdhE in this mutant could be strain-dependent because it has the *purL* gene affected by the effect of transposon insertion, whereas the *purL* gene is not affected in the wt or the clinical strains.

In summary, we have found a downregulation of Tn263 in the above mentioned proteins (with the exception of AdhE), all of which are related to the development and maintenance of the biofilm. Presumably, stress conditions caused by purine deficiency led to DNA damage in Tn263, which reduced its translational activity.

At the transcriptional level, the corresponding genes of these 13 proteins were analysed revealing interesting observations. The levels of expression of *lptD, pta, elbB, ptsI, cysJ* and *ilvC* genes were higher in the Tn263 compared to the wt strain. Interestingly, *elbB* gene was overexpressed significantly in the Tn263 strain ($p < 0.001$). This overexpression can be caused by a high level of oxidative stress, which increases the production of glyoxals [67]. The last gene related to metabolic pathways, *pta,* showed increased transcriptional levels in the mutant strain, suggesting that it could use anaerobic metabolic pathways to synthesize ATP. Transcriptional changes related to the genetic information processing group showed lower levels of *dnaK* ($p < 0.05$) and *groL* ($p < 0.01$) in the Tn263 compared to the wt strain. These data are consistent with previous observations that show the induction of expression of some genes in the *E. coli* biofilm, such as *dnaK* [18]. The *ptsI* gene expression is reduced in the wt strain. As PtsI serves as a gateway for phosphoenolpyruvate (PEP), it is likely that the transcription of PtsI might be reduced once PEP is transported through PtsI. In contrast, the slower metabolism of the mutant strain may still contain PEP that could be transported by PtsI, whose mRNA remains active. The *adhE* gene had lower expression in the Tn263 mutant than in the wt strain. The transcription of this gene is induced under anaerobic conditions and regulated at the transcriptional and translational levels by NADH/NAD$^+$ and RNase III, respectively. For this reason, the mRNA transcripts remain at low rates in Tn263 by this regulation at transcriptional and translational levels [65,68]

Finally, to detect whether these transcriptional changes were related to a biofilm-deficient phenotype, the genes with statistical significance were tested on biofilm-forming and non-biofilm-forming clinical strains. The results show disparity with those found in wt and Tn263. All genes reported equal expression in either group of clinical isolates. Among these nine genes, only two were significant (*adhE* and *pta*) and *pta* was the only one that followed the same pattern for both Tn263 and the non-biofilm-forming strains. This may be due to the insertion of the transposon in *purL* rather than being associated with a specific biofilm-forming or non-biofilm forming phenotype.

In conclusion, a mutation in the *purL* gene causes defective biofilm formation, which is related to the inability to form curli fibers, thus suggesting that this gene is essential for biofilm formation in *E. coli*. The proteomic study revealed pathways that belong to expressed biofilm factors, although the *E. coli* mutant was not able to form a biofilm. Given this reduction in biofilm formation and the influence of the biofilm on chronic infections, the identification of the *purL* gene as a target gene would

contribute to understanding of the mechanisms of biofilm production and to develop new antibiofilm treatments against biofilm-related infections caused by this microorganism.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/9/774/s1, Table S1: Biofilm classification of mutants. Low biofilm former (LF), high biofilm former (HF), biofilm former (F), Table S2: Primers used in the disruption and complementation of the *purL* gene, Table S3: Antibiotic resistance profiles of biofilm-forming and non-biofilm-forming clinical isolates, Table S4: qPCR primers used in this study, Table S5: Protein identification analysed by liquid chromatography coupled to mass spectrometry, Figure S1: Swimming assay. Figure S2: Two dimensional SDS-PAGE images of the wild type strain, Figure S3: Two dimensional SDS-PAGE images of the Tn263 mutant.

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Manuscript 1. Antimicrobial and antibiofilm activity of a fruit extract containing proteolytic enzymes

Authors: Victoria Ballén, Neus Otero, Yaiza Gabasa, Carlos Ratia, Sara M. Soto

Hypothesis: New molecules of natural origin could have a bactericidal and antibiofilm effect, helping combat antibiotic resistance and biofilm formation.

Objectives: To evaluate the antibacterial and antibiofilm activity of a fruit extract containing proteolytic enzymes against various Gram-positive and Gram-negative bacteria. (Because the extract is in the process of being patented, we cannot disclose its trade name or the fruit from which it is derived).

Materials and methods: To assess the antimicrobial capacity, the minimum inhibitory concentration (MIC) values and time-kill curve assays were determined against different biofilm-producers bacterial strains (*K.pneumoniae, E. faecalis, E. coli, S. mutans, Shigella* sp., and *Salmonella* sp.). MIC values were determined using Steer's replicator agar dilution method, and time-kill curve tests were performed using multiples of the determined MICs (0.5x, 1x, 2x, and 4x) and counting viable cells at specific time points (0, 2, 4, 8, and 24 h). In addition, the minimum biofilm eradication concentration (MBEC) was determined after 24 h of submitting the biofilm to different concentrations of the fruit extract for 24 h. In addition, to characterise the cytotoxic effect of the fruit extract, an XTT assay was performed with the Jurkat E6.1-cell line.

Results: The MIC values ranged from 35 mg/mL to 50 mg/mL. The time-kill curves revealed that fruit extract induced bacterial death at a concentration of 2x MIC and 4x MIC after 24 hours. As for MBEC, the values obtained were variable among the species. The MBEC values ranged from 150 mg/mL to 400 mg/mL, observing the

best results on *E. faecalis* (150 mg/mL) and *Shigella* strains (200 mg/mL). The half-maximal inhibitory concentration (IC_{50}) of the fruit extract was 35.7 mg/mL (1.55 in the logarithmic scale), with a cell viability of 1.6 OD. However, the value obtained could be biased due to the viscosity and turbidity of the fruit extract, leading to an inaccurate measurement, which likely hinders the XTT colourimetric assay that indicates true metabolic activity. In addition, although we got great results, it could be even better if we had isolated the active molecule rather than the whole fruit extract.

Conclusions: The fruit extract, which contains a proteolytic enzyme, showed antimicrobial and antibiofilm activity against various Gram-positive and Gram-negative strains. Thus, the investigated extract could be used in the future for the treatment of oral biofilm-associated diseases as well as intestinal infections. However, this antimicrobial activity could be improved if the active compound were isolated and tested.

1 # Antimicrobial and antibiofilm activity of a fruit extract
2 # containing proteolytic enzymes
3

4 **Victoria Ballén; Neus Otero; Yaiza Gabasa; Carlos Ratia, Sara M.
5 Soto*.**
6 ISGlobal, Hospital Clínic - Universitat de Barcelona, Barcelona, Spain.
7
8 * **Correspondence:** sara.soto@isglobal.org
9

10 Keywords: antibiofilm, antimicrobial, biofilm, fruit extract.
11

12 ## Abstract

13 Antimicrobial resistance is one of the highest public health challenges
14 worldwide of our time. Moreover, the ability of some of these bacteria
15 to form biofilms worsens the situation. Therefore, searching for new
16 molecules to counteract this critical problem is essential. In this regard,
17 new compounds such as fruit extracts are currently being studied.
18 The aim of this study was to test the antimicrobial and antibiofilm
19 properties of a fruit extract that has enzymatic activity. (Due to the
20 extract being patented, we cannot disclose its trade name or the fruit
21 from which it is derived).
22 To assess the antimicrobial capacity, the minimum inhibitory
23 concentration (MIC) values and time-kill curve assays were determined
24 against different bacterial strains (*Klebsiella pneumoniae, Enterococcus
25 faecalis, Escherichia coli, Streptococcus mutans, Shigella sp.*, and *Salmonella
26 sp.*) with the ability to form biofilms MIC values were determined using

Steer's replicator agar dilution method, and time-kill curve tests were performed using multiples of the determined MICs (0.5×, 1×, 2×, and 4×) and counting viable cells at specific time points (0, 2, 4, 8, and 24 h). In addition, the minimum biofilm eradication concentration (MBEC) was determined after 24 h of submitting the biofilm to different fruit extract concentrations.

The MIC values ranged from 35 mg/mL to 50 mg/mL. The time-kill curves revealed that fruit extract induced bacterial death at concentrations of 2× MIC and 4× MIC after 24 hours of exposure. For MBEC, the values obtained were variable among the species, ranging from 150 mg/mL to 400 mg/mL, obtaining the best results on *E. faecalis* (150 mg/mL) and *Shigella* (200 mg/mL) strains.

The results showed that the fruit extract under study has antimicrobial and antibiofilm activity. Considering that we used the total extract and not the isolated active compound, this extract could be the source of a new promising antimicrobial and antibiofilm agent.

Introduction

Antimicrobial resistance (AMR) is a global threat that claims 700,000 lives each year, with a far more significant burden in low- and middle-income countries (Pokharel et al., 2019). The spread of AMR in healthcare facilities and the community reduces the availability of effective antibiotic therapies, complicating many infections and even leading to patient death (Friedman et al., 2016).

In addition, some bacteria can form biofilms, which are aggregates of microbial cells embedded in a self-produced matrix of extracellular

52 polymeric substances (Flemming et al., 2016). Biofilms can tolerate
53 antimicrobials at concentrations 10 to 1,000 times higher than those
54 required to kill planktonic bacteria, making them very difficult to
55 eradicate (Friedman et al., 2016).

56 Biofilms are a major health problem in hospitals, causing approximately
57 50% of nosocomial infections. It becomes particularly critical in medical
58 devices such as prosthetic heart valves, pacemakers, joint prostheses,
59 intravascular catheters, endotracheal tubes, and contact lenses, which
60 create a perfect environment for bacterial adhesion (Flemming et al.,
61 2016; Lebeaux et al., 2014; Von Eiff et al., 2005).

62 In addition, biofilms have been associated with infections such as
63 dental caries, one of the most common biofilm-dependent oral
64 infectious diseases affecting hundreds of people worldwide, with *S.*
65 *mutans* being one of the most common cariogenic agents (Lin et al.,
66 2022). In addition, some *Enterobacterales*, *E. faecalis*, and other pathogens
67 have been linked with periodontitis (Espíndola et al., 2021; Gonçalves
68 et al., 2007).

69 Furthermore, biofilm-related diseases of the gallbladder and gut caused
70 by *Salmonella* sp. and *Shigella* sp. have been reported. In the case of
71 *Salmonella*, they can form biofilms in the intestine after ingestion by the
72 host. After access to the liver, *Salmonella enterica* serovar Typhi can enter
73 the gallbladder and form biofilms on cholesterol gallstones. Biofilms
74 provide *Salmonella* with protection from the gallbladder environment,
75 including bile salts and the immune system, allowing it to survive in
76 the host. Thus, chronic gallstone biofilm carriers can excrete S. Typhi
77 for at least one year or more (Harrell et al., 2021). Similarly, previous

78 studies have shown that *S. flexneri* also forms biofilms in the presence
79 of bile salts (Köseoglu et al., 2019), and acute *Shigella* infections, such as
80 intestinal biofilms, are associated with colorectal cancer and
81 inflammatory bowel disease (Chiang et al., 2021).
82 Thus, biofilms allow bacteria to survive in the body due to the difficulty
83 of eradicating the entire community using the available antibiotics.
84 Although several antimicrobial compounds can reduce the extent of the
85 biofilm, complete eradication of the bacterial community would require
86 antimicrobial concentrations above the toxicity threshold (Roy et al.,
87 2018).
88 Currently, many researchers are focusing their studies on the
89 development of new antimicrobial agents to combat AMR and
90 eradicate biofilms. One of the new approaches to achieving this goal is
91 the use of fruit extracts, which are known to contain proteolytic
92 enzymes responsible for protein degradation (Baidamshina et al., 2021;
93 Richardson et al., 2018).
94 For this reason, this study aimed to analyse the antimicrobial and
95 antibiofilm activity of a fruit extract containing a proteolytic enzyme
96 against bacterial pathogens that play a key role in biofilm-associated
97 diseases.

98 Materials and methods

99 *Bacterial strains*

100 Twenty strains of *Klebsiella pneumoniae* (n = 3), *Enterococcus faecalis* (n =
101 4), *Escherichia coli* (n = 4), *Streptococcus mutans* (n = 3), *Salmonella* sp. (n =

102 3) *and Shigella* sp. (n = 3) were used in this study. The isolates were
103 previously identified by matrix-assisted laser desorption ionisation–
104 time-of-flight mass spectrometry (MALDI-TOFF).

105 ***Determination of the minimum inhibitory concentration (MIC)***

106 This test was performed according to the agar dilution procedure
107 described in the M07-A9 guide from the Clinical and Laboratory
108 Standards Institute (CLSI, 2012). Briefly, the fruit extract was added to
109 Oxoid Iso-Sensitest™ agar at different concentrations. The bacterial
110 inocula were prepared from a fresh agar plate, and the suspension was
111 adjusted to achieve turbidity equivalent to a 0.5 McFarland standard. The
112 inoculum was diluted 1:10 in sterile saline. Aliquots from tubes
113 containing 10^7 CFU/mL were placed in the appropriate wells of a Steers
114 replicator. The replicator then dispensed 2 μL, resulting in a final
115 bacterial concentration of 10^4 CFU/mL. The plates were incubated at 37
116 °C for 24 h. Plates without extract were used to monitor bacterial
117 growth.

118 ***Time-kill curves***

119 This test provides information on the type of antibiotic inhibitory effect,
120 whether it is bactericidal or bacteriostatic. The *E. coli* ATCC 25922 strain
121 was used in this test. Different concentrations of the fruit extract were
122 tested concerning the previously determined MIC values (MIC, 2× MIC,
123 4× MIC, 8× MIC). The bacterial inoculum was adjusted to a 0.5
124 McFarland standard and diluted in Oxoid Iso-Sensitest™ broth to
125 achieve a concentration of 1×10^7 CFU/mL. Finally, 250 μL of the

126 inoculum was added to 5 mL tubes containing the appropriate
127 concentrations of fruit extract to achieve a final bacterial concentration
128 of $5x10^5$ CFU/mL. Ampicillin was used as a control due to its
129 bactericidal effect.
130 Aliquots of the liquid cultures were drawn at 0, 2, 4, 8, and 24 hours.
131 Dilutions (1:10) of the aliquots were prepared and spread on LB agar
132 plates (Condalab). The plates were incubated overnight at 37 °C, and
133 the CFU/mL were calculated. Oxoid Iso-Sensitest™ broth without
134 inoculum was used as a negative control, and broth with bacterial
135 inoculum but without fruit extract was used as a growth control.
136 Killing curves were constructed by plotting Log_{10} CFU/mL versus time.
137 Bactericidal activity was defined as a reduction of 3 or more Log_{10}
138 CFU/mL compared to the original inoculum. The bacteriostatic activity
139 was defined as the maintenance of the initial inoculum concentration
140 or a reduction of less than 3 Log_{10} CFU/mL of the total number of
141 CFU/mL (Silva et al., 2011).

142 ***Biofilm formation and quantification***

143 *S. mutans* strains were grown in BD Columbia agar containing 5% sheep
144 blood (Beckton Dickinson) at 37 °C overnight. Then, a single colony of
145 each strain was grown in 5 mL tryptic soy broth (TSB) (Condalab) at 37
146 °C overnight with shaking at 180 rpm. The overnight cultures were then
147 diluted at 1:100 in fresh medium supplemented with 1% sucrose. Then,
148 150 µL of the dilution was inoculated into polystyrene microtitre plates
149 (Nunc™ Edge 2.0 96-well plate, untreated, with lid, VWR
150 International). Culture medium without inoculum was used as a

151 sterility control. The plates were incubated for 24 h at 37 °C under static
152 conditions. Each strain was tested five times.
153 This protocol was repeated with the other species but with some
154 modifications. *K. pneumoniae* was grown in LB broth and then diluted
155 in fresh LB broth supplemented with 0.25% glucose; *E. faecalis* was
156 grown in TSB and then diluted in fresh TSB supplemented with 1%
157 glucose; *E. coli* was grown in LB broth (Condalab) and then diluted
158 1:100 in M63 medium supplemented with 0.25% glucose; *Salmonella*
159 was grown in 1/20 diluted TSB; and *Shigella* was grown in TSB
160 supplemented with 0.25% glucose and 0.4% bile salts.
161 After incubation, the biofilm biomass was quantified using crystal
162 violet staining. Optical density (OD) was measured with a microplate
163 reader (EPOCH 2 microplate reader; BioTek, VT) at a wavelength of 580
164 nm and finally interpreted according to the criteria of Stepanović et al.
165 (Stepanović et al., 2007).
166 Thus, the strains were classified as nonbiofilm formers (OD $\leq$ 0.150),
167 weak biofilm formers ($\geq$ 0.151 OD $\leq$ 0.300), moderate biofilm formers ($\geq$
168 0.301 OD $\leq$ 0.60), or strong biofilm formers (OD $\geq$ 0.601) (Ballén et al.,
169 2022).

170 ***Determination of minimum biofilm eradication concentration***
171 ***(MBEC)***

172 To evaluate the effects of the fruit extract on mature biofilms, the
173 minimum biofilm eradication concentration (MBEC) was determined.
174 After 24 h of incubation, the medium was removed, and the wells were
175 carefully washed with 200 μL of 1× PBS. Then, concentrations of the

176 fruit extract ranging between 10 mg/mL and 400 mg/mL were added to
177 each well. The microtiter plates were incubated at 37 °C for 24 h. Each
178 plate contained a negative control (culture medium without inoculum)
179 and a positive control (culture medium with bacterial inoculum).
180 After 24 h of incubation, the treatment was removed, the wells were
181 washed twice with 200 µL of 1× PBS, and the biofilm was resuspended
182 in 200 µL of 1× PBS and scraped off the plate. Tenfold dilutions were
183 performed and plated onto LB agar for colony counting.

184 ***In vitro cytotoxicity assay and data analysis***

185 The cytotoxicity of the fruit extract was established using the Jurkat
186 E6.1-cell line. Cells were maintained in RPMI-1470 media
187 supplemented with 10% fetal bovine serum (FBS), penicillin, and
188 streptomycin at 37 °C and 5% CO_2 atmosphere prior to testing. Jurkat
189 cells were grown in 96-well tissue culture plates to a cell density of 10^4
190 – 10^5 cells/well. Concentrations of the fruit extract ranging between 10
191 mg/mL and 400 mg/mL were added to the wells and incubated for 24 h
192 at 37 °C and 5% CO_2. After incubation, 50 µL XTT (Canvax, CA031) was
193 added to each well and incubated for 4 h at 37 °C and 5% CO_2.
194 The absorbance was measured at wavelengths of 450-500 nm (signal
195 absorbance) and 630-690 nm (background absorbance) using a
196 microplate reader to obtain the normalised absorbance. This value was
197 calculated by subtracting the background absorbance from the signal
198 absorbance.

199

200 $$A = 450\text{-}500 \text{ nm (Test)} - 450\text{-}500 \text{ nm (Blank)} - 630\text{-}690 \text{ nm (Test)}$$

201 A sigmoidal curve was obtained in the analysis, which is the result of
202 the calculation via a four-point logistic function to estimate the half-
203 maximal inhibitory concentration (IC_{50}). The confidence interval used
204 was 95% (CI 95%).

205 **Results**

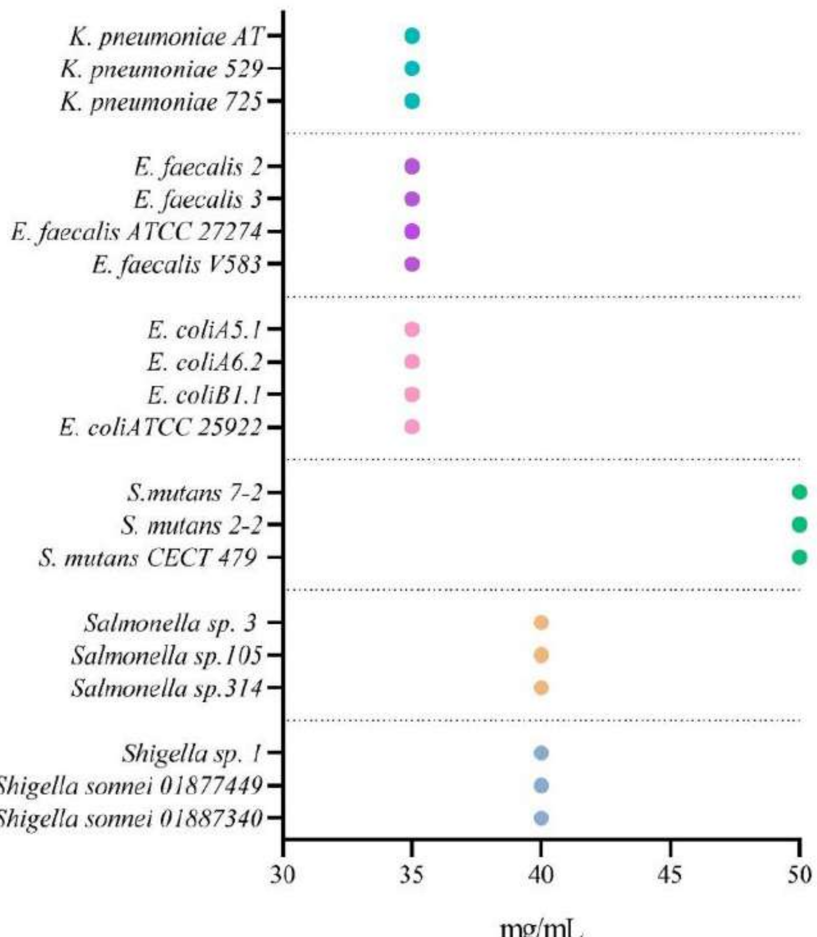
206 ***Minimal inhibitory concentration (MIC)***

207 The MIC values represent the lowest concentration of fruit extract that
208 inhibits bacterial growth. The MIC value for the *K. pneumoniae, E.*
209 *faecalis,* and *E. coli* strains was 35 mg/mL. For *Salmonella* and *Shigella,* all
210 strains showed a MIC of 40 mg/mL. Finally, all *S. mutans* strains showed
211 a MIC value of 50 mg/mL (**Figure 1**). No intraspecies differences were
212 observed in MIC, but slight interspecies differences were found.

213 ***Time-kill curves***

214 Time-dependent killing assays were performed to assess the
215 antimicrobial activity of the fruit extract. The assay was performed
216 using the fruit extract (**Figure 2A**) and ampicillin as a control (**Figure**
217 **2B**).

218 Both treatments resulted in bacterial death at 4× MIC concentrations,
219 the fruit extract after 24 h and ampicillin after 8 hours of incubation.
220 Furthermore, ampicillin 2× MIC showed the same effect as 4× MIC. It
221 was observed that ampicillin at MIC and 0.5× MIC concentrations
222 reduced bacterial counts after 4, 8, and 24 hours of treatment.

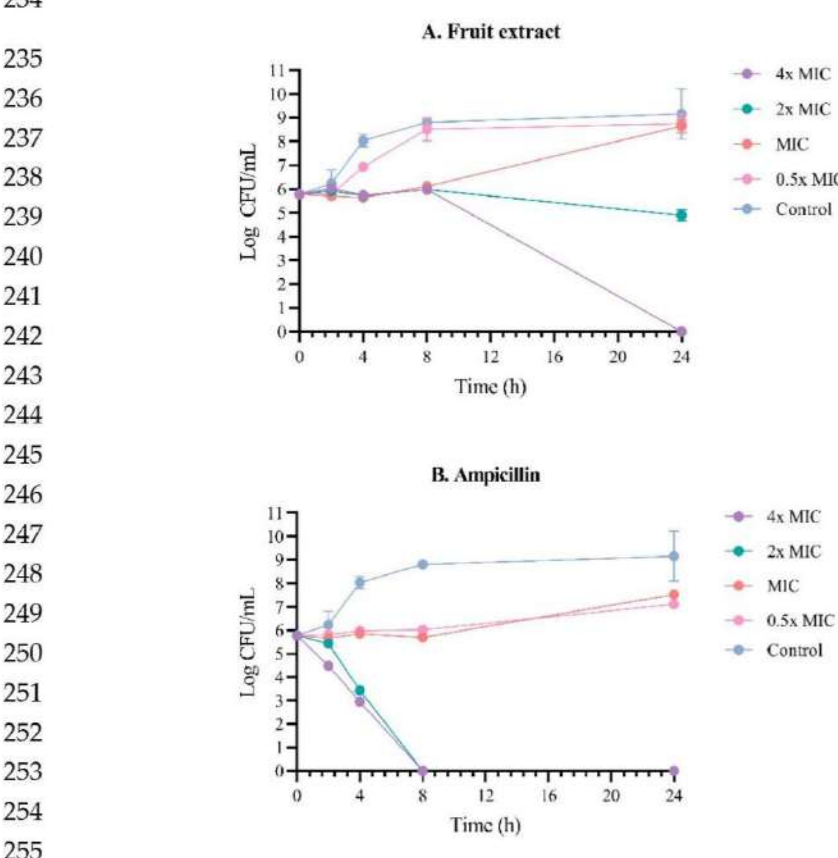


223 **Figure 1. MIC values**

224

225 In the case of the fruit extract, 2× MIC reduced colony counts at all time
226 points tested, showing a reduction of 3 Log at 24 h. However, MIC
227 concentrations reduced the count after 4 and 8 hours, but an increase in
228 the colony count was observed after 24 hours, reaching a colony count
229 similar to the control. 0.5× MIC did not show any significant effect on
230 the colony count. Thus, the fruit extract has a bactericidal effect at 24
231 hours using 2× MIC and 4× MIC concentrations. Ampicillin has a

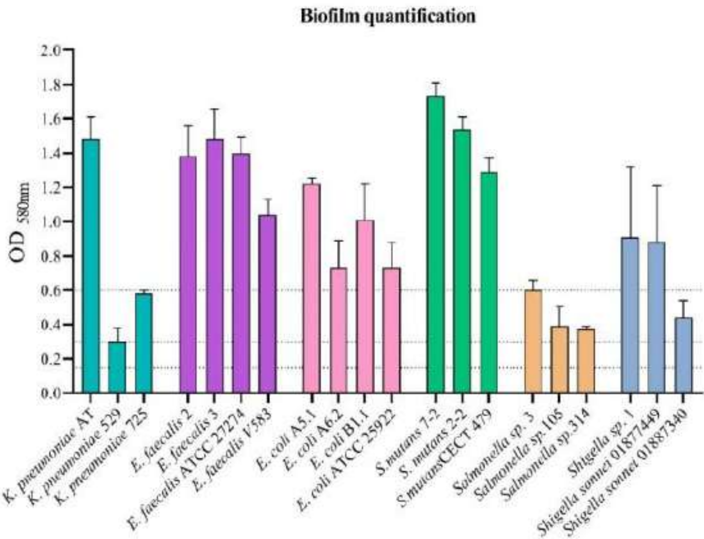
232 bactericidal effect at 4, 8, and 24 hours at 2× MIC and 4× MIC
233 concentrations.
234



257 **Figure 2. Time kill curves. A. Fruit extract; B. Ampicillin.**
258

259 ***Minimum biofilm eradication concentration (MBEC)***

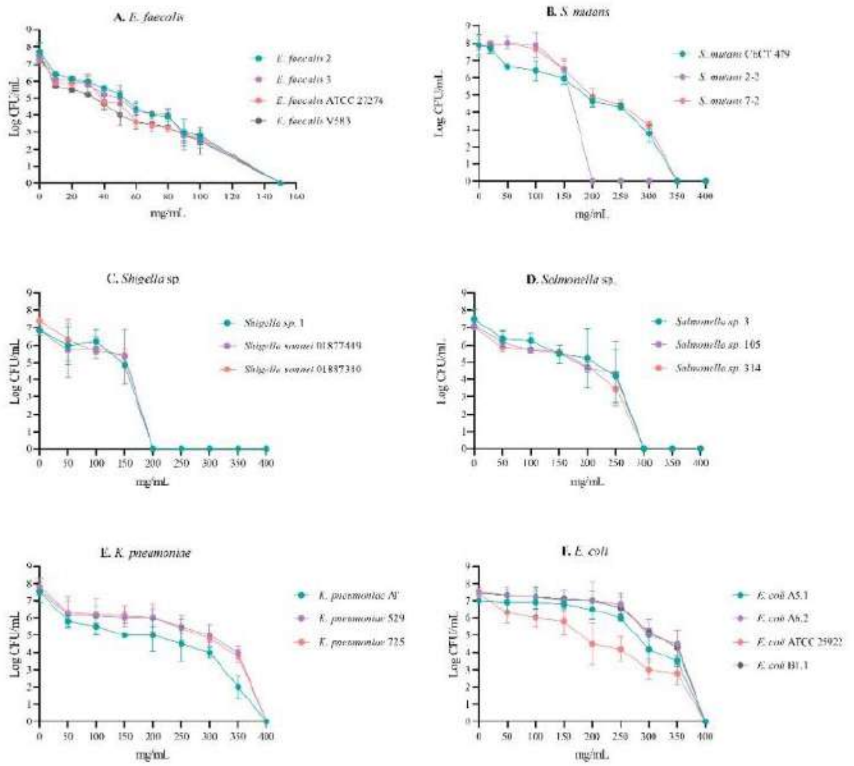
260 The biofilm quantification and the resulting classification of the strains
261 are summarised in **Figure 3**.



262 **Figure 3. Biofilm quantification**. The dotted lines indicate the cut-off
263 points for each biofilm category [nonbiofilm formers (OD ≤ 0.150), weak
264 biofilm formers (≥ 0.151 OD ≤ 0.300), moderate biofilm formers (≥ 0.301
265 OD ≤ 0.60), or strong biofilm formers (OD ≥ 0.601)].
266

267 To determine whether the fruit extract was able to eradicate biofilms,
268 the MBEC test was performed. The best results were obtained with *E.*
269 *faecalis*, where the colony count decreased by one or two logarithms at
270 a concentration of 10 mg/mL. The colony count decreased with
271 increasing extract concentration, and complete disruption was
272 observed at 150 mg/mL (**Figure 4A**). In the case of *S. mutans*, the biofilm
273 formed by strain 2-2 was disrupted at 200 mg/mL. However, the
274 biofilms formed by the other two strains, 7-2 and CECT 479, were
275 disrupted with 350 mg/mL fruit extract (**Figure 4B**).

276 Among the Gram-negative species studied, the best results were
277 obtained in *Shigella* strains, showing MBEC values of 200 mg/mL
278 (**Figure 4C**). The MBEC values of all *Salmonella* strains were 300 mg/mL
279 (**Figure 4D**). The results obtained in *K. pneumoniae* (**Figure 4E**) and *E.*
280 *coli* (**Figure 4F**) were very similar, showing complete biofilm disruption
281 at 400 mg/mL.



282

283 **Figure 4. Minimum biofilm eradication concentration (MBEC) of the**
284 **fruit extract. A.** *E. faecalis*; **B.** *S. mutans*; **C.** *Shigella* sp.; **D.** *Salmonella* sp.;
285 **E.** *K. pneumoniae*; **F.** *E. coli*.

286 ***Determination of the fruit extract IC_{50} by XTT colorimetric assay***

287 The cytotoxic effect of the fruit extract was determined by the XTT assay
288 using Jurkat E6.1 cells. This assay measured the metabolic activity of
289 the cells and thus assessed their viability. The IC_{50} of the fruit extract
290 was 35.7 mg/mL (1.55 in the logarithmic scale), with a cell viability of
291 1.6 OD (**Figure 5**). This value provides information on the concentration
292 required to inhibit a specific biological or biochemical process *in vitro*
293 by 50%.

294

295

296

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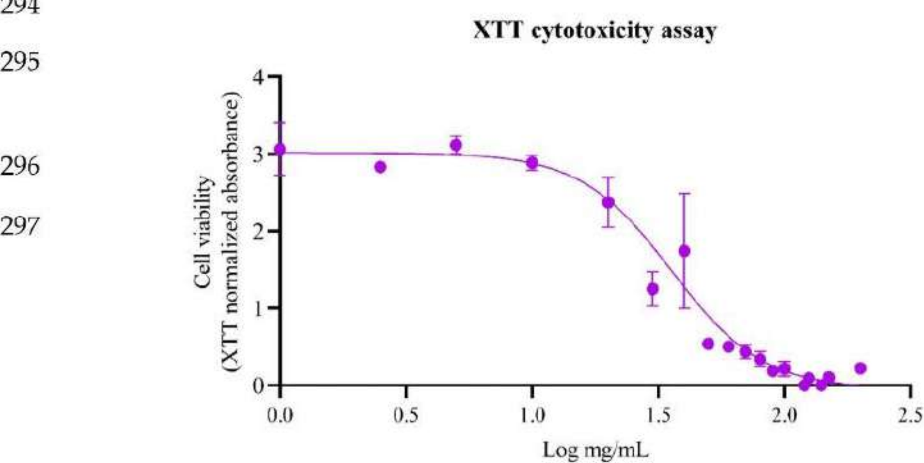

298 **Figure 5. IC_{50} determination of fruit extract by XTT cytotoxicity assay**.

299

300 **Discussion**

301 Biofilm formation is associated with higher antimicrobial resistance
302 than planktonic forms (Donlan, 2001). This fact is a challenge and a
303 public health concern; therefore, the development of new molecules to
304 combat AMR and eradicate biofilms is needed (Bjarnsholt et al., 2018;
305 Singhal et al., 2019). However, as the underlying mechanisms of

306 biofilm formation are not fully understood, the discovery of new
307 antibiofilm agents is an obstacle for the scientific community (Cepas et
308 al., 2019).

309 This study investigated the antibacterial and antibiofilm properties of a
310 fruit extract with proteolytic activity against some Gram-positive and
311 Gram-negative bacteria. We found that this extract could be considered
312 a promising molecule to achieve this goal due to the excellent MIC and
313 MBEC values obtained. In addition, we also demonstrated that the
314 extract was bactericidal at 2× MIC and 4× MIC after 24 hours of
315 incubation.

316 One of the essential approaches to eradicate biofilms is to target their
317 structural components. In this sense, several enzymes, including some
318 proteases, can actively disperse biofilms by degrading the EPS matrix
319 (Jiang et al., 2020; Srinivasan et al., 2021). For example, serine protease
320 Esp, secreted by *Staphylococcus epidermidis*, inhibits biofilm formation
321 and destroys mature *S. aureus* biofilms (Shukla & Rao, 2017). In
322 addition, cysteine proteases secreted by equine mesenchymal stromal
323 cells (MSCs) have been shown to destabilise methicillin-resistant *S.*
324 *aureus* biofilms (Marx et al., 2020).

325 Similarly, proteolytic enzymes from fruits are currently being
326 investigated as promising new molecules for biofilm eradication.
327 Papain is an important plant peptidase with potent proteolytic activity
328 derived from papaya fruit (*Carica papaya*) (Eshamah et al., 2014).
329 Baidamshina et al. have shown that papain can destroy biofilms and
330 present synergistic effects with existing antibiofilm agents
331 (Baidamshina et al., 2021). In addition, the study by Mugita et al. found

332 that actinidin, a cysteine protease from kiwi, significantly removed the
333 tongue coating in elderly subjects, and trypsin, papain, and actinidin
334 reduced *in vitro* monospecies and multispecies biofilms (Mugita et al.,
335 2017). Furthermore, several studies have shown that the antibacterial
336 effect of some proteolytic enzymes can be enhanced by combining
337 them with conventional antibiotics (Bjarnsholt et al., 2018; Li et al.,
338 2015).

339 Thus, the use of matrix-degrading enzymes, such as those analysed in
340 this study, could be a potential technique for various biofilm-related
341 infections. For example, due to their excellent pH stability and catalytic
342 activity in the oral cavity, proteases are efficient in preventing caries
343 without disturbing the biological balance in the oral cavity (Lin et al.,
344 2022).

345 However, it is important to note that our study has some limitations.
346 First, the IC_{50} value obtained could be biased due to the viscosity and
347 turbidity of the fruit extract, leading to an inaccurate measurement,
348 which likely hinders the XTT colorimetric assay that indicates the real
349 metabolic activity. In addition, although we obtained great results, they
350 could be improved if we could study the isolated active molecule rather
351 than the whole fruit extract. Future studies may allow the isolation of
352 the active compound from the extract to further analyse the
353 antimicrobial and antibiofilm activity of this promising molecule alone
354 and in combination with conventional antibiotics with possible
355 synergistic activity.

356 ## Conclusions

357 The fruit extract under study, which contains a proteolytic enzyme,
358 showed antimicrobial and antibiofilm activity against various Gram-
359 positive and Gram-negative species. Thus, due to the range of species
360 inhibited, the investigated extract could be used in the future for the
361 treatment of oral biofilm-associated diseases as well as intestinal
362 infections. However, this antimicrobial activity could be improved if
363 the active compound were isolated and tested.

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376 ## Author contributions

377 SS and VB: conceptualisation and writing—original draft preparation. VB, NO,
378 and YG: methodology. VB, CR, and SS: writing—review and editing. All
379 authors have read and agreed to the published version of the manuscript.

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519

Results

Paper 3. Antibiotic Resistance and Virulence Profiles of *Klebsiella pneumoniae* Strains Isolated from Different Clinical Sources

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Authors: Victoria Ballén, Yaiza Gabasa, Carlos Ratia, Raquel Ortega, Marc Tejero and Sara Soto

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Hypothesis: The origin of the bacterial strains, their antimicrobial resistance profiles, and the presence or absence of different virulence genes could be related to biofilm formation.

Objectives: To characterise a collection of clinical strains of *E. coli* and *K. pneumoniae* for antimicrobial resistance and virulence determinants to elucidate a relationship between these characteristics and the ability to form biofilms.

Materials and methods: In this study, 127 strains from different clinical sources (urine, respiratory tract, or blood) were characterised for antimicrobial resistance, the presence of virulence factor genes, serum resistance, hypermucoviscosity, and the ability to form biofilms. Specific characteristics of the uropathogenic strains were examined and compared with the other clinical groups.

Results: Some differences were found between the strain isolated from urine and the other groups of strains. Urine strains showed the highest antibiotic resistance (64.91%) compared to blood (63.64%) or respiratory strains (51.35%), as well as the highest extended-spectrum beta-lactamases (ESBL) production. These strains also

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showed statistically significant high resistance to fosfomycin (24.56%) compared to the other groups ($p = 0.008$). Regarding virulence, 84.21% of the urine strains presented the *uge* gene, showing a statistically significant difference ($p = 0.03$) compared to the other clinical sources, indicating a possible role of this gene in developing the urinary tract infection. In addition, 46% of biofilm-forming strains belonged to the urine sample group ($p = 0.043$).

Conclusions: We found that isolates from urine were more resistant to antimicrobials, were more ESBL-producers and were more biofilm-formers than respiratory and blood strains. Our study also correlated the presence of the *uge* gene with UTIs and the presence of yersiniabactin with respiratory tract infections. In addition, although we found an inverse relation between antimicrobial resistance and virulence, the acquisition of mobile genetic elements could promote the spread of antimicrobial resistance genes and that of virulence genes that evolve toward pathotypes considered to be more virulent. The increasing coexistence of these two conditions is of particular concern as it can lead to untreatable and invasive *K. pneumoniae* infections. Active surveillance for antimicrobial resistance and virulence determinants is imperative to avoid the transmission and spread of hypervirulent or extensively resistant strains.

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Antibiotic Resistance and Virulence Profiles of *Klebsiella pneumoniae* Strains Isolated From Different Clinical Sources

*Victoria Ballén, Yaiza Gabasa, Carlos Ratia, Raquel Ortega, Marc Tejero and Sara Soto**

ISGlobal, Hospital Clínic—Universitat de Barcelona, Barcelona, Spain

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***Correspondence:**
Sara Soto
sara.soto@isglobal.org

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Klebsiella pneumoniae is a Gram-negative bacterium capable of colonizing, invading, and causing infections in different anatomical sites of the human body. Its ability to evade the immune system, its increasing antimicrobial resistance and the emergence of hypervirulent pathotypes have become a major challenge in the medical field. In this study, 127 strains from different clinical sources (urine, respiratory tract or blood) were characterized for antimicrobial resistance, the presence of virulence factor genes, serum resistance, hypermucoviscosity and the ability to form biofilms. Specific characteristics of the uropathogenic strains were examined and compared with the other clinical groups.

Differences were found between urine and the other groups of strains. Urine strains showed the highest antibiotic resistance (64.91%) compared to blood (63.64%) or respiratory strains (51.35%) as well as the highest extended-spectrum beta-lactamases (ESBL) production. These strains also showed statistically significant high resistance to fosfomycin (24.56%) compared to the other groups ($p = 0.008$). Regarding virulence, 84.21% of the urine strains presented the *uge* gene, showing a statistically significant difference ($p = 0.03$) compared to the other clinical sources, indicating a possible role of this gene in the development of urinary tract infection. In addition, 46% of biofilm-forming strains belonged to the urine sample group ($p = 0.043$). In conclusion, *K. pneumoniae* strains isolated from urine samples showed higher antimicrobial resistance, ESBL production, and biofilm-forming ability compared to those isolated from respiratory or blood samples. The rapid spread of clinical strains with these characteristics is of concern, and new therapeutic alternatives are essential to mitigate their harmful effects.

Keywords: *Klebsiella pneumoniae*, antimicrobial resistance, virulence, biofilm, urinary tract infections

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen associated with a variety of infections, being urinary tract infections (UTIs) among the most common worldwide. *K. pneumoniae* is considered an important uropathogen in ambulatory patients (Foxman, 2010). Although UTIs do not result in a high mortality rate, they represent a significant economic burden to the health care system as they increase treatment costs.

However, *K. pneumoniae* is not only responsible for UTIs but also for respiratory and bloodstream infections (Podschun and Ullmann, 1998). It is one of the species recognized as part of the ESKAPE group, associated by their characteristic potential to escape or evade the action of antimicrobial agents. This acronym comprises six highly virulent and antibiotic-resistant bacterial pathogens including *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. Additionally, the World Health Organization (WHO) lists *K. pneumoniae* as one of the species of high priority and promotes the research and development of new antibiotics due to the growing global problem of antimicrobial resistance (World Health Organization, 2017).

Uropathogenic bacteria have some characteristics that favor the colonization of human cells, such as the production of adhesins, siderophores, and toxins (Foxman, 2010). In the same way, these bacteria can adhere to medical devices forming biofilms, avoiding the immune system and favoring the antimicrobial therapy failure (Dybowska-Sarapuk et al., 2017). For this reason, the characterization of *K. pneumoniae* antimicrobial resistance, its ability to form biofilm and its virulence are key to understanding the pathogenicity of this bacteria in the clinical setting and to achieve more efficient antimicrobial therapy.

The aim of this study was to characterize a collection of *K. pneumoniae* strains isolated from different clinical sources (urine, respiratory tract, or blood) in terms of antimicrobial resistance, the presence of virulence factor genes, serum resistance, hypermucoviscosity, and the ability to form biofilms. The specific characteristics of the uropathogenic strains were studied and compared to *K. pneumoniae* strains isolated from respiratory and/or blood samples.

MATERIALS AND METHODS

Bacterial Strains

One hundred twenty-seven *K. pneumoniae* clinical strains from four Catalonian hospitals (Hospital Clinic de Barcelona, Hospital Universitario de Bellvitge, Hospital del Mar, and Hospital Universitario Mutua de Terrassa) were collected over six months from 2016 to 2017. Among these, 57 strains were obtained from urine (1 urinary catheter, 1 suprapubic aspiration, and 55 midstream urine), 37 from respiratory samples (7 sputum, 10 bronchoalveolar aspirates, 4 tracheal samples, 1 bronchoalveolar lavage, 15 non-classified respiratory samples), and 33 strains were obtained from blood. Strains were identified as *K. pneumoniae* using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik GmbH, Bremen, Germany). The modified score values suggested by the manufacturer were used: A score ≥2.3 meant species identification; a score between 2.0 and 2.299 meant genus identification and probable species identification; a score between 1.7 and 1.9 meant probable genus identification; and a score <1.69 meant non-reliable identification. Strains with a score ≥2.3 classified as *K. pneumoniae* were included in the study. The strains were stored in skim milk (BD) at -80°C. All data on the strains used in this study can be found in the **Supplementary Material**.

Determination of Antimicrobial Resistance

Determination of antimicrobial resistance was assessed by disk diffusion or broth microdilution (in the case of colistin) methods following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2020). The antimicrobial agents tested by Kirby-Bauer method were amoxicillin/clavulanate (20/10 µg), aztreonam (30 µg), cefepime (30 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), fosfomycin (200 µg/50 µg of glucose-6-phosphate), gentamicin (10 µg), imipenem (10 µg), piperacillin/tazobactam (100/10 µg), and trimethoprim-sulfamethoxazole (1.25/23.75 µg). *E. coli* ATCC 25922 was used as quality control.

Strains were classified according to their antimicrobial resistance profile as susceptible, resistant to 1 or 2 antimicrobial categories, multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR). The European Centre for Disease Prevention and Control (ECDC) proposed the MDR, XDR, and PDR definitions. An isolate is considered MDR if it is non-susceptible to at least 1 agent in ≥3 antimicrobial categories, defined as XDR if the isolate is non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories, and PDR if the isolate is non-susceptible to all listed antimicrobial agents (Magiorakos et al., 2012).

Extended-spectrum beta-lactamases (ESBL) production was evaluated by the ESBL test and carbapenemase production was analyzed by modified carbapenem inactivation and EDTA-modified carbapenem inactivation methods following CLSI guidelines (CLSI, 2020).

String Test

The string test was performed to identify the hypermucoviscous (HMV) phenotype. The strains were grown in BD Columbia agar with 5% sheep blood (Beckton Dickinson) at 37°C overnight. Afterwards, a bacteriological inoculation loop was used to stretch a mucous bacterial colony. The HMV phenotype was positive when a string > 5 mm in length was observed (Fang et al., 2004).

Determination of Virulence and Antimicrobial Resistance Genes

A single colony from an overnight culture plate was selected and suspended in 100 µL of sterilized Milli-Q water. The suspension was boiled for 10 min and centrifuged for 10 min at 13000 rpm. The supernatant was used as the DNA template.

Polymerase chain reaction (PCR) was performed to detect different virulence genes: fimbriae (*fimD, fimH, mrkC, mrkD*),

capsule-associated genes (*ycfM*, *wabG*, *uge* and *rmpA*), siderophores (*entB*, *iucA*, *irp2*, *ybtS*, *fyuA*, *iroN*), colibactin genes (*clbA*, *clbQ*), and capsular serotypes (K1, K2).

ESBL-associated genes bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, bla_{OXA48}, in addition to genes conferring resistance to quinolones [*aac (6)-Ib-cr*, *qnrB*], aminoglycosides (*aadb*), and sulfonamides (*sul1* and *sul2*), were detected by PCR. The primers used are listed in **Table 1**.

Biofilm Formation and Quantification

Biofilm formation was performed using the protocol described by O'Toole and Kolter, (1998) with some modifications. Briefly,

TABLE 1 | Primers to detect virulence and antimicrobial resistance genes.

Target gene	Primer sequence (5' → 3')	Melting temperature (Tm °C)	Size of amplicon (bp)	Reference
fimD – F	GTTACGCCTATCTGAATCTACAGAG	57	1114	This study
fimD – R	GACCAGTTGATATCGTCCACG			
fimH – F	GAAAAAAATAATCCCCCTGTTCAC	57	855	This study
fimH – R	GTAACCTGGCCTGTGGTC			
mrkC – F	GCTCAACTCCATCGTCAGC	57	1056	This study
mrkC – R	CGCGAGTTATTGAACGAGGTG			
mrkD – F	CGCTTTTTATCGTCTTAATG	55	880	This study
mrkD – R	GTGATGTAGCGGGTCTCCTG			
ycfM – F	ATCAGCAGTCGGGTCAGC	57	160	(Candan and Aksöz, 2015)
ycfM – R	CTTCTCCAGCATTCAGCG			
entB – F	ATTTCCTCAACTTCTGGGGC	60	371	(Candan and Aksöz, 2015)
entB – R	AGCATCGGTGGCGGTGGTCA			
iucA – F	AATCAATGGCTATTCCCGCTG	58	239	(Garza-Ramos et al., 2018)
iucA – R	CGCTTCACTTCTTTCACTGACAGG			
irp2 – F	GCTACAATGGGACAGCAACGAC	58	230	(Garza-Ramos et al., 2018)
irp2 – R	GCAGAGCGATACGGAAAATGC			
ybtS – F	GACGGAAACAGCACGGTAAA	53	242	(Compain et al., 2014)
ybtS – R	GAGCATAATAAGGCGAAAGA			
fyuA – F	TTTCCACCAACACCATCCAG	58	817	This study
fyuA – R	CAGGTCAGGTCACTGTATGC			
iroN – F	AACCGCCAGATCGATATTCG	58	827	This study
iroN – R	TTAATCTCACCGCTGGTTCG			
rmpA – F	CATAAGAGTATTGGTTGACAG	56	461	(Compain et al., 2014)
rmpA – R	CTTGCATGAGCCATCTTTCA			
wabG – F	CGGACTGGCAGATCCATATC	58	683	(Jian-li et al., 2017)
wabG – R	ACCATCGGCCATTTGATAGA			
uge – F	GATCATCCGGTCTCCCTGTA	59	535	This study
uge – R	TCTTCACGCCTTCCTTCACT			
K1 – F	GGTGCTCTTTACATCATTGC	59	1283	(Lin et al., 2014)
K1 – R	GCAATGGCCATTTGCGTTAG			
K2 – F	GACCCGATATTCATACTTGACAGAG	59	641	(Lin et al., 2014)
K2 – R	CCTGAAGTAAAATCGTAAATAGATGGC			
clbA – F	CTAGATTATCCGTGGCGATTC	51	1002	(Morgan et al., 2019)
clbA – R	CAGATACACAGATACCATTCA			
clbQ – F	CTTGTATAGTTACACAACTATTTC	50	821	(Morgan et al., 2019)
clbQ – R	TTATCCTGTTAGCTTTCGTTC			
*bla*SHV-1 – F	ATGCGTTATATTCGCCTGTG	49	747	(Wiegand et al., 2007)
*bla*SHV-1 – R	TGCTTTGTTATTCGGGCCAA			
*bla*TEM-1 - F	TCGCCGCATACACTATTCTCAGAATGA	53	445	(Monstein et al., 2007)
*bla*TEM-1 - R	ACGCTCACCGGCTCCAGATTTAT			
*bla*CTX-M1 - F	AAAAATCACTGCGCCAGTTC	52	415	(Woodford et al., 2006)
*bla*CTX-M1 - R	AGCTTATTCATCGCCACGTT			
*bla*OXA48 - F	TTGGTGGCATCGATTATCGG	57	744	(Queenan and Bush, 2007)
*bla*OXA48 - R	GAGCACTTCTTTTGTGATGGC			
aac(6')-Ib-cr - F	TTGCGATGCTCTATGAGTGGCTA	60	482	(Park et al., 2006)
aac(6')-Ib-cr - R	CTCGAATGCCTGGCGTGTTT			
qnrB – F	GATCGTGAAAGCCAGAAAGG	52	469	(Robicsek et al., 2006)
qnrB – R	ACGATGCCTGGTAGTTGTCC			
aadb – F	ATGGACACAACGCAGGTCGC	55	534	(Guo et al., 2016)
aadb – R	TTAGGCCGCATATCGCGACC			
sul1 - F	CTTCGATGAGAGCCGGCGGC	63	436	(Guerra et al., 2004)
sul1 - R	GCAAGGCGGAAACCCGCGCC			
sul2 - F	TCAACATAACCTCGGACAGT	55	707	(Guerra et al., 2004)
sul2 - R	GATGAAGTCAGCTCCACCT			

isolates were cultured in Luria Bertani (LB) agar (Miller's LB AGAR, Condalab) for 24 h at 37°C. Then, a single colony of each strain was grown in 10 mL of LB broth (LB, Condalab) for 24 h at 37°C with shaking at 180 rpm. Afterwards, overnight cultures were diluted 1:100 in fresh medium supplemented with 0.25% glucose, 200 µL were inoculated in polystyrene microtiter plates (Nunc™ Edge 2.0 96-well plate, non-treated, with lid, VWR International), and incubated for 48 h at 37°C in static conditions. Culture medium without inoculum was used as a sterility control.

After incubation, the liquid of each well was removed, washed once with 1x phosphate-buffered saline (PBS), and dried at 65°C. Plates were then stained with crystal violet (CV) (2% v/v) for 10 min at room temperature. Next, plates were washed once with 1x PBS and fixed at 65°C for 60 min. The CV was detached by the addition of 33% glacial acetic acid and biomass was calculated measuring the optical density (OD) at 580 nm using a microplate reader (EPOCH 2 microplate reader, BioTek, VT). The experiment was carried out in three technical and biological replicates.

The strains were classified according to the criteria of Stepanović et al. (2007) as: non-biofilm formers (OD ≤ 0.150), weak biofilm formers (≥ 0.151 OD ≤ 0.300), moderate biofilm formers (≥ 0.301 OD ≤ 0.60), or strong biofilm formers (OD ≥ 0.601).

Serum Resistance

Serum resistance was analyzed following the procedure described by (Podschun et al., 2016). Briefly, 25 µL of inoculum of 2.5×10^6 colony forming units (CFU)/mL of a mid-log phase culture were mixed with 75 µL of normal human serum obtained from healthy volunteers in 96-well polystyrene, round-bottomed microtiter plates (Greiner bio-one). The mixture was incubated at 37°C for 1, 2, and 3 h. After incubation, the cell count was determined using 10-fold serial dilutions and conventional plating in LB agar (Miller's LB AGAR, Condalab). The test was graded as shown in **Table 2**. The assay was performed in triplicate.

Statistical Analysis

Statistical analysis was performed using the Chi-square test with the IBM SPSS Statistics software for Windows, version 21.0. *P*-values < 0.05 were considered statistically significant.

TABLE 2 | Classification of serum resistance.

	Cell counts/mL (compared to the original inoculum)		
	1 h	2 h	3 h
Grade 1	<10%	<10%	<0.1%
Grade 2	10% - 100%	10% - 100%	<10%
Grade 3	>100%	<100%	<100%
Grade 4	>100%	>100%	<100%
Grade 5	>100%	>100%	>100% (decreasing)
Grade 6	>100%	>100%	>100% (increasing)

RESULTS

Antimicrobial Resistance

Overall, 50 isolates (39.37%) were susceptible to all the antimicrobial agents tested, 24 isolates (18.90%) were non-susceptible to 1 or 2 categories of antibiotics, 51 isolates (40.16%) were considered multidrug-resistant (MDR), and 2 strains (1.57%) were considered extensively drug-resistant (XDR). None was classified as pandrug-resistant (**Figure 1**). The percentages of resistance for each antibiotic tested are shown in **Figure 2**.

Among uropathogenic *K. pneumoniae*, we found 20 susceptible strains (35.09%), 12 strains (21.05%) were non-susceptible to 1 or 2 categories, and 25 were MDR strains (43.86%). The uropathogenic group showed the highest overall antibiotic resistance (64.91%) compared to blood (63.64%) and respiratory tract (51.35%) strains, although these differences were not statistically significant. The highest percentages of antimicrobial resistance were observed for ciprofloxacin (42.11%), amoxicillin/clavulanic acid (36.84%), trimethoprim-sulfamethoxazole (36.84%) and ceftazidime (33.33%). Uropathogenic *K. pneumoniae* also showed the highest resistance to fosfomycin (24.56%) with the difference being statistically significant (p = 0.008) compared to the other groups.

Among the respiratory group, 18 strains (48.65%) were classified as susceptible, 6 strains (16.22%) as non-susceptible to 1 or 2 categories, 11 strains (29.73%) as MDR, and 2 strains (5.40%) as XDR. The highest percentages of antimicrobial resistance were observed with amoxicillin/clavulanic acid (40.54%), ciprofloxacin (37.84%), piperacillin/tazobactam (37.84%) and ceftazidime (35.14%). In comparison to the other groups, respiratory strains showed the highest resistance to imipenem (24.32%) and piperacillin/tazobactam (37.84%), being these differences statistically significant (p = 0.003 both).

Strains isolated from blood samples were classified as follows: 12 strains (36.36%) were susceptible, 6 strains (18.18%) were non-susceptible to 1 or 2 categories, and 15 strains (45.46%) were MDR. The highest percentages of antimicrobial resistance were observed with ceftazidime (45.45%), followed by ciprofloxacin, amoxicillin/clavulanic acid and trimethoprim-sulfamethoxazole with a percentage of resistance of 42.42% each.

Presence of Antimicrobial Resistance Genes

Fifty-five isolates (43.31%) were ESBL producers. Among these, 24 (43.6%) were isolated from urine, 16 (29.1%) from blood, and 15 (27.3%) from the respiratory tract. The gene most frequently detected was bla_{SHV-1} (85.45%), followed by bla_{CTX-M1} (78.95%), and bla_{TEM-1} (71.93%). Thirteen strains (10.24%) were carbapenemase producers: 9 from the respiratory tract (69.2%), 4 from blood (30.8%) and none from urine. Among these, 10 (76.92%) harbored the bla_{OXA48} gene. Concerning the 52 (40.94%) strains resistant to ciprofloxacin, 20 (38.46%) harbored the *aac (6')-Ib-cr* gene, and 27 (51.92%) harbored the *qnrB* gene. One strain among the group of gentamicin-resistant strains harbored the *aadb* gene. Regarding the 44 strains classified as resistant to trimethoprim-sulfamethoxazole (34.65%), six (13.64%) and 29 (65.91%) carried the *sul1* and the *sul2* genes, respectively.

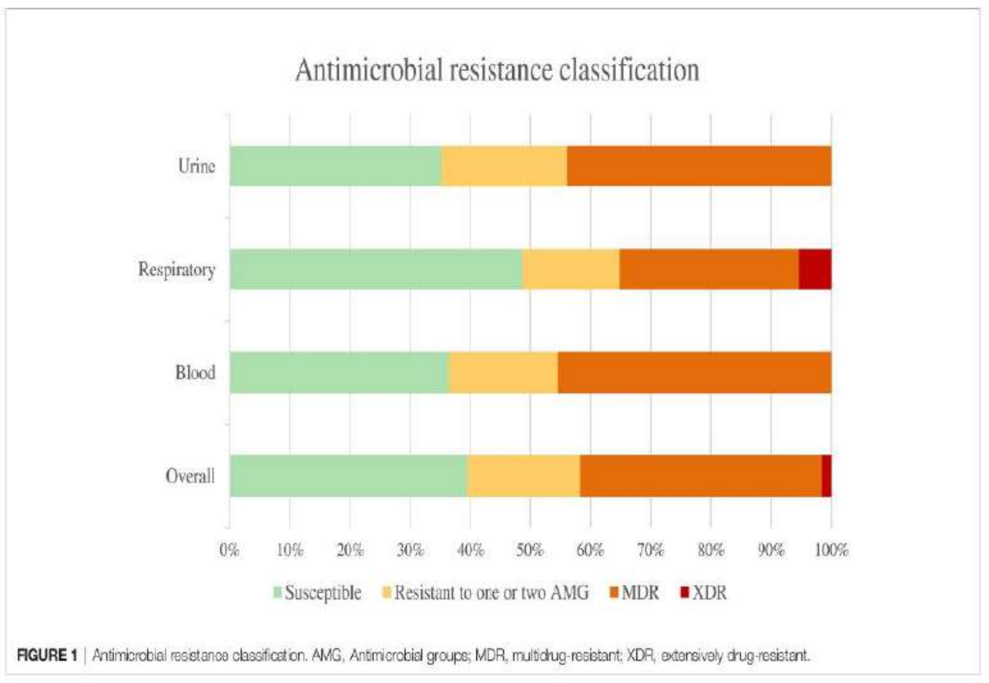
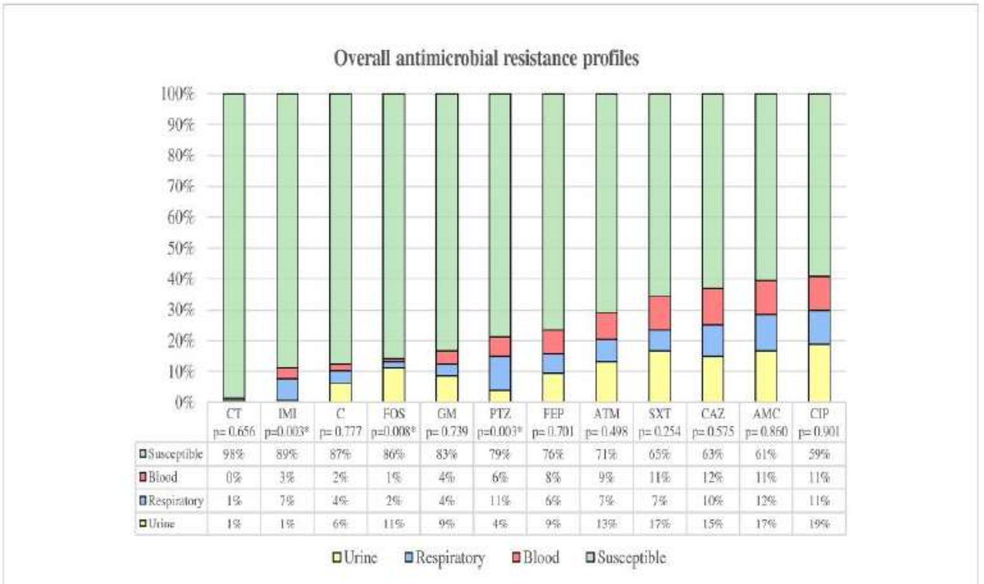


FIGURE 1 | Antimicrobial resistance classification. AMG, Antimicrobial groups; MDR, multidrug-resistant; XDR, extensively drug-resistant.



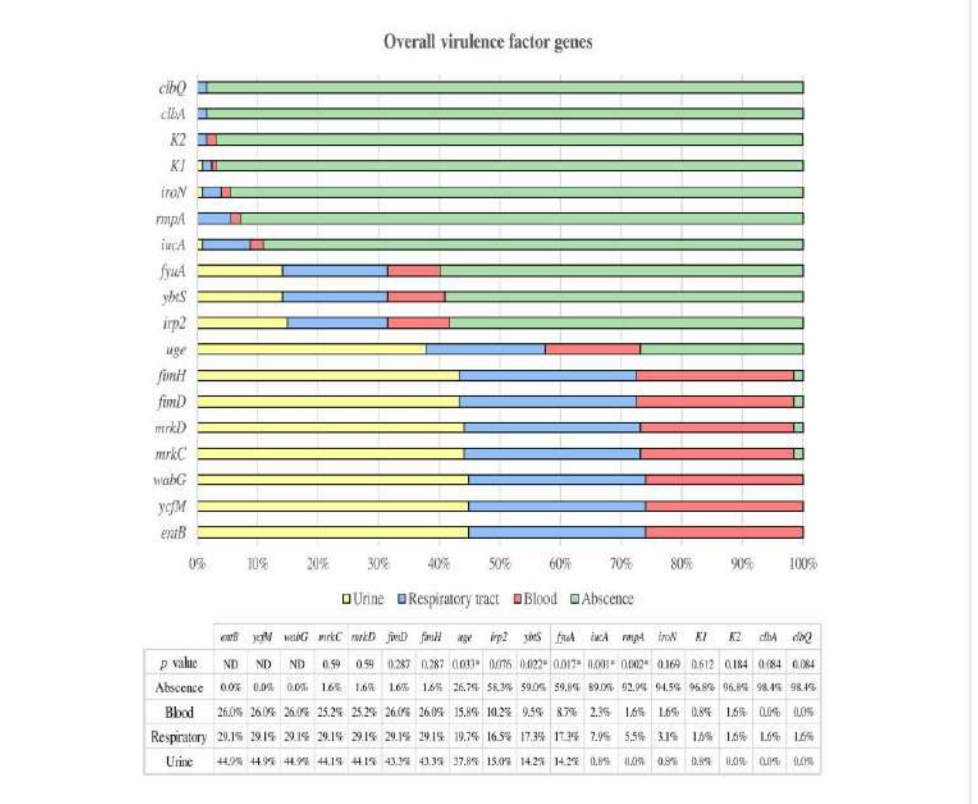
	CT p= 0.656	IMI p=0.003*	C p= 0.777	FOS p=0.008*	GM p= 0.739	PTZ p=0.003*	FEP p= 0.701	ATM p= 0.498	SXT p= 0.254	CAZ p= 0.575	AMC p= 0.860	CIP p= 0.901
Susceptible	98%	89%	87%	86%	83%	79%	76%	71%	65%	63%	61%	59%
Blood	0%	3%	2%	1%	4%	6%	8%	9%	11%	12%	11%	11%
Respiratory	1%	7%	4%	2%	4%	11%	6%	7%	7%	10%	12%	11%
Urine	1%	1%	6%	11%	9%	4%	9%	13%	17%	15%	17%	19%

FIGURE 2 | Overall antimicrobial resistance profiles were categorized by clinical source. Susceptibility rate includes all clinical sources, while resistance rates are classified by urine, respiratory tract, and blood. * Statistically significant ($p < 0.05$). CT, colistin; IMI, imipenem; C, chloramphenicol; FOS, fosfomycin; GM, gentamicin; PTZ, piperacillin/tazobactam; FEP, cefepime; ATM, aztreonam; SXT, trimethoprim-sulfamethoxazole; CAZ, ceftazidime; AMC, amoxicillin/clavulanate; CIP, ciprofloxacin.

Presence of Virulence Genes

The results obtained are shown in **Figure 3**. All the isolates carried the *ycfM*, *entB*, and *wabG* genes. The *fimD*, *fimH*, *mrkC*, and *mrkD* genes were almost ubiquitous among the strains (98.43%). The prevalence of the other virulence genes was as follows: *uge* (73.23%), *irp2* (41.73%), *ybtS* (40.94%), *fyuA* (40.16%), *iucA* (11.02%), *rmpA* (7.09%), *iroN* (5.51%), *clbA* (1.57%), and *clbQ* (1.57%).

Among all these genes, the *uge* gene showed a higher prevalence among urinary strains compared to respiratory and blood samples, being the difference statistically significant ($p = 0.033$). On the other hand, the *iucA*, *ybtS*, *fyuA* and *rmpA* genes were statistically more prevalent in respiratory isolates than urinary or blood strains ($p = 0.001$, $p = 0.022$, $p = 0.017$, and $p = 0.002$, respectively).



	entB	*ycfM*	*wabG*	*mrkC*	*mrkD*	*fimD*	*fimH*	*uge*	*irp2*	*ybtS*	*fyuA*	*iucA*	*rmpA*	*iroN*	*K1*	*K2*	*clbA*	*clbQ*
p value	ND	ND	ND	0.59	0.59	0.287	0.287	0.033*	0.076	0.022*	0.017*	0.001*	0.002*	0.169	0.612	0.184	0.084	0.084
Absecence	0.0%	0.0%	0.0%	1.6%	1.6%	1.6%	1.6%	26.7%	58.3%	59.0%	59.8%	89.0%	92.9%	94.5%	96.8%	96.8%	98.4%	98.4%
Blood	26.0%	26.0%	26.0%	25.2%	25.2%	26.0%	26.0%	15.8%	10.2%	9.5%	8.7%	2.3%	1.6%	1.6%	0.8%	1.6%	0.0%	0.0%
Respiratory	29.1%	29.1%	29.1%	29.1%	29.1%	29.1%	29.1%	19.7%	16.5%	17.3%	17.3%	7.9%	5.5%	3.1%	1.6%	1.6%	1.6%	1.6%
Urine	44.9%	44.9%	44.9%	44.1%	44.1%	43.3%	43.3%	37.8%	15.0%	14.2%	14.2%	0.8%	0.0%	0.8%	0.8%	0.0%	0.0%	0.0%

FIGURE 3 | Overall virulence factor genes were categorized according to the clinical source. All strains in which the respective gene is not present are assigned to the "absence" category, while the presence of the gene is classified according to the clinical source (urine, respiratory and blood). * Statistically significant ($p < 0.05$). ND, Not determined.

String Test and Serum Resistance

In the present study, 17 *K. pneumoniae* strains (13.39%) were identified as hypermucoviscous by the string test. Among these, 7 strains were isolated from urine, 7 from the respiratory tract and 3 from blood.

Table 3 shows the results of serum resistance. We observed that more than 50% of the strains tested were highly resistant to serum, but there was no correlation between this test and the other characteristics under study (type of isolate, biofilm formation, hypermucoviscosity, antimicrobial resistance classification, or the presence of virulence genes).

Biofilm Formation and Quantification

Twenty-five strains (19.69%) were classified as non-biofilm formers, 35 (27.55%) as weak biofilm formers, 42 (33.07%) as moderate biofilm formers, and 25 strains (19.69%) as strong biofilm formers. Among the 102 biofilm-forming strains, including weak, moderate and strong, 47 (46.1%) were from urine, 30 (29.4%) were from blood, and 25 strains (24.5%) were from the respiratory tract (**Figure 4**). When grouping the strains into biofilm-forming and non-biofilm-forming, we found that the highest number of biofilm-forming strains were from urine, and compared to the other groups of isolates, the difference was statistically significant (p = 0.043).

TABLE 3 | Results of serum resistance test.

	Serum resistance			
	Urine	**Respiratory**	**Blood**	**Overall**
	57 (44.88%)	37 (29.13%)	33 (25.99%)	127 (100%)
Grade 1	18 (31.58%)	7 (18.92%)	6 (18.18%)	31 (24.41%)
Grade 2	6 (10.53%)	6 (16.22%)	3 (9.09%)	15 (11.81%)
Grade 3	3 (5.26%)	1 (2.70%)	0 (0%)	4 (3.15%)
Grade 4	1 (1.75%)	0 (0%)	0 (0%)	1 (0.79%)
Grade 5	2 (3.51%)	0 (0%)	1 (3.03%)	3 (2.36%)
Grade 6	27 (47.37%)	23 (62.16%)	23 (69.70%)	73 (57.48%)

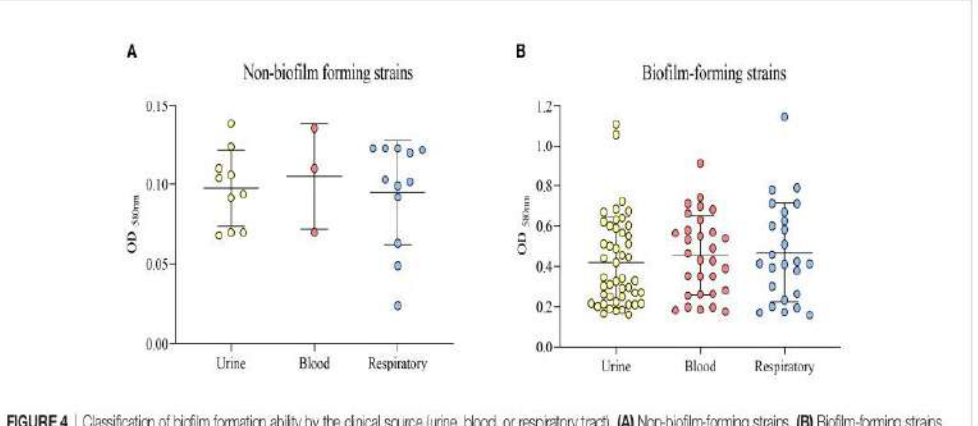


FIGURE 4 | Classification of biofilm formation ability by the clinical source (urine, blood, or respiratory tract). **(A)** Non-biofilm-forming strains. **(B)** Biofilm-forming strains.

Relationship Between Antimicrobial Resistance and Virulence

There was a statistically significant relationship between the strains carrying the *iucA*, *rmpA* and *uge* genes and the strains classified as susceptible in the antimicrobial resistance classification ($p = 0.028$; $p = 0.006$; $p < 0.001$).

Excluding colistin, we observed a statistically significant relationship between the presence of the *uge* gene and the susceptibility to the other antibiotics tested ($p < 0.005$). Likewise, strains harboring the *rmpA* gene were more susceptible to amoxicillin/clavulanate, ciprofloxacin, trimethoprim-sulfamethoxazole and cefepime ($p = 0.012$; $p = 0.010$; $p = 0.023$; $p = 0.046$). Strains harboring *iroN* or *iucA* genes were more susceptible to amoxicillin/clavulanate ($p = 0.028$; $p = 0.042$) and ciprofloxacin ($p = 0.023$; $p = 0.032$). In addition, strains carrying *iroN* genes were also more susceptible to trimethoprim-sulfamethoxazole ($p = 0.048$).

A similar relationship was found between the strains carrying the *irp2* gene and susceptibility to ceftazidime and gentamicin ($p = 0.020$; $p = 0.040$), and the strains harboring the *ybtS* gene and the susceptibility to gentamicin ($p = 0.009$).

The *irp2*, *ybtS*, and *fyuA* genes showed a higher prevalence among ESBL-producing strains ($p < 0.001$, $p = 0.02$, and $p = 0.012$, respectively), and the *rmpA* and the *uge* genes were more prevalent among the non-ESBL-producing strains ($p = 0.043$ and $p < 0.001$, respectively). Nevertheless, the *irp2*, *ybtS* and *uge* genes showed a higher prevalence among non-carbapenemase-producer strains ($p = 0.034$; $p = 0.029$; $p = 0.003$).

Concerning the hypermucoviscous phenotype, the lack of the *iucA*, *iroN*, and *rmpA* genes was related to the non-hypermucoviscous phenotype ($p < 0.05$ in all cases). Hypermucoviscous strains also showed a lower capacity to form biofilm ($p = 0.017$) and a higher susceptibility to gentamycin and amoxicillin/clavulanate ($p = 0.049$ both).

There were significant differences between biofilm-forming and non-biofilm-forming isolates concerning the *iucA*, *iroN*, and *rmpA* genes, with biofilm-forming strains lacking these genes ($p = 0.02$, $p = 0.01$, and $p < 0.001$, respectively).

In the case of uropathogenic strains, we found a statistically significant association between the presence of the *irp2*, *ybtS* and *fyuA* genes and susceptibility to fosfomycin ($p = 0.017$, $p = 0.024$, and $p = 0.024$, respectively). Similarly, we found significant associations between the presence of the *uge* gene and susceptibility to aztreonam, cefepime, ceftazidime, chloramphenicol, ciprofloxacin and trimethoprim-sulfamethoxazole ($p = 0.002$, $p = 0.008$, $p < 0.001$, $p = 0.004$, $p = 0.018$ and $p = 0.043$, respectively). On the other hand, we found that the strains lacking the *iucA* gene were more susceptible to imipenem ($p < 0.001$) and chloramphenicol ($p = 0.013$), whereas the strains lacking the *iroN* gene were more susceptible to piperacillin/tazobactam ($p = 0.001$).

Among the respiratory strains, we found a statistically significant association between the presence of the *iucA* gene and susceptibility to imipenem ($p = 0.036$), piperacillin/tazobactam ($p = 0.034$), amoxicillin/clavulanate ($p = 0.021$), aztreonam ($p = 0.006$), cefepime ($p = 0.036$), ciprofloxacin ($p = 0.004$) and trimethoprim-sulfamethoxazole ($p = 0.036$). Similarly, we found a significant association between the presence of the *rmpA* gene and susceptibility to piperacillin/tazobactam ($p = 0.022$), amoxicillin/clavulanate ($p = 0.025$), aztreonam ($p = 0.031$) and ciprofloxacin ($p = 0.022$). The presence of the *uge* gene was correlated with susceptibility to aztreonam ($p = 0.005$) and ciprofloxacin ($p = 0.012$). However, strains lacking the *ybtS*, *irp2* or *fyuA* genes were more susceptible to gentamicin ($p = 0.047$ in all cases). Finally, strains carrying the *ybtS* gene were more resistant to imipenem ($p = 0.039$).

For the group of strains isolated from blood, we found a significant association between the presence of the *uge* gene and susceptibility to amoxicillin/clavulanate ($p = 0.001$), aztreonam ($p < 0.001$), cefepime ($p < 0.001$), ceftazidime ($p < 0.001$), chloramphenicol ($p = 0.024$), ciprofloxacin ($p < 0.001$), gentamicin ($p = 0.003$) and trimethoprim-sulfamethoxazole ($p = 0.012$). Nevertheless, strains lacking the *iucA* or *ybtS* genes were more susceptible to chloramphenicol ($p = 0.024$ and $p = 0.016$). Similarly, strains lacking the *irp2* gene were more susceptible to cefepime ($p = 0.044$) and gentamicin ($p = 0.044$). Finally, strains carrying the *irp2* gene were more resistant to aztreonam ($p = 0.027$).

DISCUSSION

K. pneumoniae causes a wide range of infections and uses different virulence factors to colonize and spread in the human body. The increasing antimicrobial resistance of this bacteria in recent years is of great concern to the scientific community.

In this study, we characterized a collection of 127 clinical *K. pneumoniae* strains isolated from urine, the respiratory tract, and blood and compared these strains according to antimicrobial resistance and the presence of a wide variety of virulence factors.

Overall, we found that more than 40% of isolates were classified as MDR and almost half of these were from urine. The data from the 2017 European Antimicrobial Resistance Surveillance Network report showed that at the European Union and the European economic area level, more than one third of *K. pneumoniae* isolates were resistant to at least one of the antimicrobial groups under regular surveillance. The report showed resistance percentages for fluoroquinolones (31.5%), followed by third-generation cephalosporins (31.2%), aminoglycosides (24.1%), and carbapenems (7.2%) (European Centre for Disease Prevention and Control (ECDC), 2018) similar to the general percentages observed in this study.

Urine isolates showed the highest overall antibiotic resistance (64.91%), closely followed by strains isolated from blood (63.64%) and the respiratory tract (51.35%). Uropathogenic isolates made up the majority of the ESBL-producing strains. Compared to the multicenter study carried out for Garcia-Fernandez et al., our strains showed a higher prevalence of ESBL and lower carbapenemase production. This previous study reported a prevalence of 23.1% of ESBL-producing *Klebsiella* spp. and 20% of carbapenemase-producing *Klebsiella* (García-Fernández et al., 2019). This is of great concern because some antibiotic resistance genes are carried by mobile genetic elements that facilitate horizontal genetic exchange and promote the spread of antimicrobial resistance genes within and between species. Their characterization in future studies could help us to expand our research. On the other hand, multidrug resistance has been associated with increased health care costs, longer hospital stays, and high mortality rates. Moreover, nowadays there are few new alternatives for effective treatments.

Urine strains also showed the highest resistance rates to fosfomycin (24.56%). Fosfomycin trometamol is one of the antibiotics proposed for treating uncomplicated UTIs, and its consumption has increased by 20.7% since 2012 in Spain, according to the Joint Inter-agency Antimicrobial Consumption and Resistance Analysis report (Alonso Herreras et al., 2018). Sorlozano et al. showed that ESBL-producing *Klebsiella* had low susceptibility rates to fosfomycin ranging between 40 and 78% (Sorlozano et al., 2014). Therefore, it is important to consider this antibiotic in the persistent monitoring and surveillance of antimicrobial resistance.

Regarding virulence, hypermucoviscous pathotypes, genes for adhesins, iron uptake and transport, toxins, and biofilm formation ability are some of the factors that *K. pneumoniae* employs in pathogenesis. Although many studies define hypervirulent *K. pneumoniae* (hvKp) as string test positive, it has been demonstrated that not all hvKp strains are hypermucoviscous (Fu et al., 2018). The study carried out by Russo et al. proposed the *rmpA* and *iucA* genes as biomarkers for distinguishing between hypervirulent (hvKp) and the classical (cKp) *K. pneumoniae* pathotypes (Russo et al., 2011). *rmpA* is a plasmid-located virulence factor gene that regulates the synthesis of capsular polysaccharides (Wang et al., 2020), and *iucA* is involved in the biosynthesis of the siderophore aerobactin (De Lorenzo and Neilands, 1986). These genes were significantly associated with the hvKp-phenotype in our study, in which seven of the 17 (41.18%) hypermucoviscous strains carried both genes. On the other hand, two more strains were found, *icuA*+, *rmpA*+ and HMV-, five were classified as *icuA*+, *rmpA*- and HMV- and ten were classified as *icuA*-, *rmpA*- and HMV+. This suggests that other regulatory mechanisms may be involved in the expression of the hypermucoviscous phenotype. Thus, considering both the string test and the hypervirulence-related genes *iucA* and *rmpA*, 24 strains (18.9%) were classified as hypervirulent in the present work. In terms of antimicrobial resistance, hvKp have been classified as susceptible or as ESBL-producer strains (Harada and Doi, 2018). We found 13 strains classified as susceptible and nine as ESBL-producers, similar to the studies previously described. Of the other two strains with an *icuA*-, *rmpA*- and HMV+ phenotype, one was classified as resistant to fosfomycin and the other as resistant to more than three categories of antibiotics.

Other hypervirulence-associated genes, such as *iroBCDN*, *iutA*, *iuacABCD*, *ybt* and the *clb* locus, may be encoded in the chromosome as part of integrative conjugative elements (Arato et al., 2021). The hypervirulent phenotype confers important properties to bacteria for pathogenesis, such as capsule polysaccharide production, phagocytosis resistance, dissemination and systemic infection in the host (Xu et al., 2021). Early identification of hvKp strains would allow better understanding of the infection and the implementation of more effective antimicrobial treatment. Likewise, it is mandatory to avoid the possible dissemination of these pathotypes by implementing barriers between patients, with precautions based on virulence likely being as important as those based on antimicrobial resistance.

Among siderophores, enterobactin (encoded by *ent* genes), aerobactin (encoded by *iuc* genes), salmochelin (encoded by *iro* genes), and yersiniabactin (encoded by *ybt* genes, regulated by *irp* genes, and the receptor is encoded by the *fyuA* gene) are the most representative in *K. pneumoniae*. Siderophores are indispensable to obtain the iron necessary for the growth and replication of bacteria. They scavenge iron from host transport proteins. Enterobactin is produced by all *K. pneumoniae*, but its mechanism may be disabled by human lipocalin-2 (Lcn2), which has an affinity for enterobactin and elicits an inflammatory response. In contrast, yersiniabactin eludes binding to Lcn2, thereby avoiding the inflammatory response and promoting bacterial growth (Lam et al., 2018). As expected, we found the *entB* gene in all the strains tested in agreement with another study (Fatima et al., 2021). Besides, the higher prevalence of the siderophore-encoded *ybtS* and *fyuA* genes in the respiratory tract isolates compared to the urinary

and blood strains confirmed that yersiniabactin is an important virulence factor for the pulmonary infection, as reported by previous studies (Yan et al., 2016).

The capsule-associated genes *wabG*, (encoding capsule), *uge* (encoding capsule lipoprotein) and *ycfM* (promoting external membrane protein) are highly involved in virulence by promoting infection through resistance to phagocytosis. We observed the presence of the *uge* (uridine diphosphate galacturonate-4 epimerase) gene in the majority of the isolates from urine, showing a statistically significant difference compared to the other clinical isolates. It has been demonstrated that *K. pneumoniae* strains lacking the *uge* gene are less virulent and less capable of causing UTI, pneumonia or sepsis. Mutations in the *uge* gene reduce the colonization ability of *K. pneumoniae* in experimentally induced urinary infections (Regué et al., 2004; Candan and Aksöz, 2015; Remya et al., 2019). However, the occurrence of the *uge* gene in *K. pneumoniae* has varied widely in different studies. Remya et al. found a prevalence of 48.6% harboring the *uge* gene. Its distribution in different clinical specimens was blood (50%), urine (48.8%), exudates (48.4%), and respiratory secretions (47.3%). El Fertas-Aissani et al. observed the presence of the *uge* gene in 84.6% of urinary tract strains and all the blood and respiratory isolates (El Fertas-Aissani et al., 2013). Although the *uge* gene has previously been described as essential for *K. pneumoniae* virulence, future research may provide further evidence for its specific association with UTIs.

Among the toxins tested, two of the strains were positive for the *clbA* and *clbQ* genes, two of the genes encoding colibactin. Colibactin is a recently described toxin that has been associated with colorectal cancer in humans because it causes DNA damage in host cells. Colibactin has been found in *E. coli*, but its occurrence in other species is steadily increasing. Acquisition of the *pks* locus, which synthesizes this toxin, has been linked to intestinal colonization and mucosal invasion by *K. pneumoniae* (Strakova et al., 2021).

Interestingly, one of the strains isolated from the respiratory tract was positive for all the virulence genes tested. Two other strains were positive for almost all the virulence genes except those encoding colibactin. These three strains were susceptible to all antimicrobial agents, and were classified as serotype K1, non-biofilm-forming, or weak biofilm-forming strains and showed high serum resistance, which protects the bacteria from phagocytosis (Hennequin and Robin, 2016). Although more research is needed on the influence of the acquisition of antibiotic resistance mechanisms by the bacteria on fitness cost and the relation with virulence, we found a scenario in which the greater the virulence, the lesser the antimicrobial resistance. Indeed, we found a correlation between the presence of some virulence factor genes and susceptibility to most of the antibiotics tested.

The genes encoding ESBL are carried by plasmids that may also harbor other genes, including some virulence factor genes. In previous studies, three virulence factors (adhesin CF29K, aerobactin or mucoid phenotype) were found in ESBL-producing *K. pneumoniae* strains. (Hennequin and Robin, 2016). In our study, we found the same association between *fyuA*, *irp2* or *ybtS* genes and ESBL-producing strains. The *ybt* carried by the FIB_K plasmid is a novel mechanism for *ybt* mobilization. Currently, many FIB_K plasmids also contain antimicrobial resistance transposons, which may indicate the coexistence of virulence and resistance genes in the same mobile genetic element (Lam et al., 2018).

Regarding biofilm, almost half of biofilm-forming strains, including weak, moderate and strong, were isolated from urine and the difference from the other clinical sources was statistically significant. *K. pneumoniae* has been identified as the second most prevalent pathogen in UTIs (Liu et al., 2020), and the ability to form biofilms on indwelling urethral catheters is one of the most common conditions, affecting thousands of people worldwide each year. Recurrence of catheter urinary tract infections after antibiotic treatment could be caused by re-colonization by bacteria that survived in the biofilm (Stahlhut et al., 2012). Besides, biofilms present higher resistance to phagocytosis and higher antimicrobial resistance compared to planktonic bacteria (Trautner and Darouiche, 2004). Nonetheless, truly effective strategies to prevent or counteract biofilm-related infections remain elusive.

CONCLUSION

We found that isolates from urine were more resistant to antimicrobials and were more ESBL-producers, and more biofilm-formers compared to respiratory and blood strains. Our study also correlated the presence of the *uge* gene with UTIs and the presence of yersiniabactin with respiratory tract infections. In addition, although we found an inverse relation between antimicrobial resistance and virulence, the acquisition of mobile genetic elements could promote not only the spread of antimicrobial resistance genes but also that of virulence genes that evolve toward pathotypes considered to be more virulent. The increasing coexistence of these two conditions is of particular concern as it can lead to untreatable and invasive *K. pneumoniae* infections. Active surveillance not only for antimicrobial resistance but also for virulence determinants is imperative to avoid the transmission and spread of hypervirulent or extensively resistant strains.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization, SS and VB. Methodology, VB, YG, MT, RO, and CR. Statistical analysis, VB. Writing-original draft preparation, VB and SS. Writing—review and editing, VB, CR,

YG, and SS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.738223/full#supplementary-material

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Results

Paper 4. Correlation between Antimicrobial Resistance, Virulence Determinants and Biofilm Formation Ability among Extraintestinal Pathogenic *Escherichia coli* Strains Isolated in Catalonia, Spain

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Authors: Victoria Ballén, Yaiza Gabasa, Carlos Ratia, Melany Sánchez and Sara Soto

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Hypothesis: The origin of the bacterial strains, their antimicrobial resistance profiles, and the presence or absence of different virulence genes could be related to biofilm formation.

Objectives: Characterise a collection of clinical strains of *E. coli* and *K. pneumoniae* for antimicrobial resistance and virulence determinants to elucidate a relationship between these characteristics and the ability to form biofilms.

Materials and methods: 376 extraintestinal pathogenic *E. coli* strains collected from four hospitals in Catalonia (Spain) between 2016 and 2017 were characterised by antimicrobial resistance, siderophore production, phylogroup classification, and the presence of selected virulence and antimicrobial resistance genes. In addition, the association between these characteristics and the ability to form biofilms was also analysed. Due to the high prevalence of colibactin among the studied strains, we analysed their clonal relationship by pulsed-field gel electrophoresis (PFGE) of XbaI-digested DNA.

Results: The strains studied were classified into four groups according to their biofilm formation ability: non-biofilm formers (15.7%), weak (23.1%), moderate (35.6%), and strong biofilm formers (25.6%). The strains were highly resistant to ciprofloxacin (48.7%), trimethoprim-sulfamethoxazole (47.9%), and ampicillin (38%), showing a correlation between higher resistance to ciprofloxacin and lower biofilm production. Seventy-three strains (19.4%) were ESBL-producers. However, no relationship between the presence of ESBL and biofilm formation was found. The virulence factor genes *fimH* (92%), *pgaA* (84.6%), and *irp1* (77.1%) were the most prevalent in all the studied strains. A statistically significant correlation was found between biofilm formation and the presence of *iroN*, *papA*, *fimH*, *sfa*, *cnf*, *hlyA*, *iutA*, and colibactin-encoding genes *clbA*, *clbB*, *clbN*, and *clbQ*. Interestingly, a high prevalence of colibactin-encoding genes (19.9%) was observed. Most colibactin-encoding *E. coli* isolates belonged to phylogroup B2, exhibited low antimicrobial resistance but moderate or high biofilm-forming ability, and were significantly associated with most of the virulence factor genes tested. Additionally, the analysis of their clonal relatedness by PFGE showed 48 different clusters, indicating a high clonal diversity among the colibactin-positive strains.

Conclusions: Ciprofloxacin resistance was associated with lower biofilm production ability among the *E. coli* strains under study. Furthermore, biofilm formation ability was correlated with some siderophores, adhesins, and toxins. The high percentage of strains harbouring the colibactin-encoding genes and the significant correlation between this toxin and biofilm formation suggest that colibactin could be a promising therapeutic target to prevent biofilm formation. Nevertheless, further studies are needed to confirm our hypothesis and better understand the impact of colibactin production on human health.

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Correlation Between Antimicrobial Resistance, Virulence Determinants and Biofilm Formation Ability Among Extraintestinal Pathogenic *Escherichia coli* Strains Isolated in Catalonia, Spain

*Victoria Ballén, Yaiza Gabasa, Carlos Ratia, Melany Sánchez and Sara Soto**

ISGlobal, Hospital Clínic—Universitat de Barcelona, Barcelona, Spain

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Edited by:
Jana Seifert,
University of Hohenheim, Germany

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Veronica Maria Jarocki,
University of Technology Sydney, Australia

***Correspondence:**
Sara Soto
sara.soto@isglobal.org

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Escherichia coli is a well-characterized bacterium highly prevalent in the human intestinal tract and the cause of many important infections. The aim of this study was to characterize 376 extraintestinal pathogenic *E. coli* strains collected from four hospitals in Catalonia (Spain) between 2016 and 2017 in terms of antimicrobial resistance, siderophore production, phylogroup classification, and the presence of selected virulence and antimicrobial resistance genes. In addition, the association between these characteristics and the ability to form biofilms was also analyzed. The strains studied were classified into four groups according to their biofilm formation ability: non-biofilm formers (15.7%), weak (23.1%), moderate (35.6%), and strong biofilm formers (25.6%). The strains were highly resistant to ciprofloxacin (48.7%), trimethoprim-sulfamethoxazole (47.9%), and ampicillin (38%), showing a correlation between higher resistance to ciprofloxacin and lower biofilm production. Seventy-three strains (19.4%) were ESBL-producers. However, no relationship between the presence of ESBL and biofilm formation was found. The virulence factor genes *fimH* (92%), *pgaA* (84.6%), and *irp1* (77.1%) were the most prevalent in all the studied strains. A statistically significant correlation was found between biofilm formation and the presence of *iroN*, *papA*, *fimH*, *sfa*, *cnf*, *hlyA*, *iutA*, and colibactin-encoding genes *clbA*, *clbB*, *clbN*, and *clbQ*. Interestingly, a high prevalence of colibactin-encoding genes (19.9%) was observed. Colibactin is a virulence factor, which interferes with the eukaryotic cell cycle and has been associated with colorectal cancer in humans. Most colibactin-encoding *E. coli* isolates belonged to phylogroup B2, exhibited low antimicrobial resistance but moderate or high biofilm-forming ability, and were significantly associated with most of the virulence factor genes tested. Additionally, the analysis of their clonal relatedness by PFGE showed 48 different clusters, indicating a high clonal diversity among the colibactin-positive strains. Several studies have correlated the pathogenicity of *E. coli* and the presence of virulence factor genes; however, colibactin and its relationship

to biofilm formation have been scarcely investigated. The increasing prevalence of colibactin in *E. coli* and other Enterobacteriaceae and the recently described correlation with biofilm formation, makes colibactin a promising therapeutic target to prevent biofilm formation and its associated adverse effects.

Keywords: ***Escherichia coli***, **antimicrobial resistance, virulence, biofilm, colibactin**

INTRODUCTION

Escherichia coli is a well-characterized bacterium which plays an essential role in the human microbiome. Nevertheless, some strains are responsible for intestinal and extraintestinal infections. Extraintestinal pathogenic *E. coli* (ExPEC) strains are commonly implicated in a variety of infectious diseases occurring in either the community or healthcare settings worldwide, resulting in high economic and social costs (Beloin et al., 2008; Blount, 2015; Manges et al., 2019).

Additionally, *E. coli* can form aggregates and attach to solid surfaces, forming complex structures called biofilms. Bacteria in biofilms secrete various components such as extracellular polymeric substances (EPS), which protect the bacterial community from host immunity and the effects of antibiotics, complicating the infection (Sharma et al., 2016).

In addition to the ability to form biofilms, the ExPEC group has many virulence factor genes (VFGs) encoding adhesins, toxins, siderophores, capsules, and invasins, which are often located into pathogenicity islands (PAIs), plasmids, and other mobile genetic elements (Sarowska et al., 2019). Some of these VFGs can promote biofilm formation. Among the well-characterized VFGs correlated with biofilm formation, type I fimbriae, curli fimbriae, and flagella are the most studied. The *fimA* gene encodes the major subunit of type I fimbriae, which are known to be involved in the first step of biofilm formation (Beloin et al., 2004). Curli fimbriae are encoded by curli-specific genes (*csg*) and are involved in adhesion to surfaces and invasion of eukaryotic host cells (Van Gerven et al., 2018). Flagella play an important role not only in cell motility but also in surface adhesion (Friedlander et al., 2015). Some studies suggest that deletion of some of these genes leads to a reduction in the ability to form biofilm (Guttenplan and Kearns, 2013; Smith et al., 2017).

One of the most recently studied VFGs is colibactin, a secondary metabolite encoded in the genomic island *pks*, which interferes with the eukaryotic cell cycle and has been linked to colorectal cancer in humans (Dziubańska-Kusibab et al., 2020). Interestingly, this PAI is commonly observed among *E. coli* strains belonging to phylogroup B2, including ExPEC, and has been found in isolates from the intestinal microbiota, septicemia, neonatal meningitis, and urinary tract infections (UTIs) (Faïs et al., 2018; Dziubańska-Kusibab et al., 2020).

The aim of this study was to characterize 376 ExPEC strains in terms of antimicrobial resistance, biofilm formation, siderophore production, phylogroup classification, and presence of selected virulence and antimicrobial resistance genes. The association between these features and the ability to form biofilm was also analyzed. Understanding the VFGs that correlate with biofilm production is needed, as these could be considered targets for developing new antimicrobial therapies.

MATERIALS AND METHODS

Bacterial Strains

A total of 376 ExPEC strains were collected from four hospitals in Catalonia, Spain, between 2016 and 2017. Of these, 132 were isolated from blood, 60 from respiratory samples (13 sputum, 12 bronchoalveolar aspirates, 4 tracheal samples, 2 pleural fluid, and 29 non-classified respiratory samples) and 184 from urine (1 from a urinary catheter and 183 from midstream urine). Strains were identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) (Bruker Daltonik GmbH, Bremen, Germany). The modified score values suggested by the manufacturer were used: a score $\geq$ 2.3 meant species identification; a score between 2.0 and 2.299 meant genus identification and probable species identification; a score between 1.7 and 1.9 meant probable genus identification; and a score $<$ 1.69 meant non-reliable identification. Only strains with a score $\geq$ 2.3 classified as *E. coli* were included in the study. After that, the strains were stored in Skim Milk (Becton Dickinson) at −80°C.

Biofilm Formation and Quantification

To determine biofilm formation, we performed a protocol previously developed by our group (Cepas et al., 2020). Briefly, strains were cultured in Luria Bertani (LB) agar (Miller's LB AGAR, Condalab) for 18 – 24 h at 37°C. Then, the cell suspension was prepared in 10 mL LB broth and incubated for 18 – 24 h at 37°C with shaking (180 rpm). After incubation, each culture was diluted 1:100 in M63 medium [13.5 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 5.0×10^{-4} g/L $FeSO_4$, 1 mL 1 M $MgSO_4.7H_2O$], supplemented with 0.25% glucose and tested in 96-well flat-bottomed non-treated polystyrene microtiter plates with lids (Nunc® Edge 2.0, VWR International, Barcelona, Spain) at 30°C for 48 h under static conditions.

The supernatant was then removed and the biofilms were washed once with 1x PBS and dried at 65°C. The plates were stained with crystal violet 2% (CV) for 10 min, washed with 1x PBS, and dried at 65°C. The CV was resuspended with glacial acetic acid 33%, and the biomass was quantified by measuring the optical density at 580 nm using a microplate reader (EPOCH 2 microplate reader; BioTek, VT). The experiment was performed in three technical and biological replicates, and the results were interpreted according to the criteria of

Stepanović et al. (2007). Thus, the strains were classified as non-biofilm formers (OD $\leq$ 0.150), weak biofilm formers ($\geq$ 0.151 OD $\leq$ 0.300), moderate biofilm formers ($\geq$ 0.301 OD $\leq$ 0.60), or strong biofilm formers (OD $\geq$ 0.601). *Escherichia coli* ATCC 25922 was used as positive control, and M63 broth without bacterial inoculum was used as negative control.

Antimicrobial Susceptibility Testing

Antimicrobial resistance profiling was performed using the most representative antimicrobial agents from the different antibiotic families, which are of great clinical and epidemiological relevance. Kirby-Bauer disk diffusion or broth microdilution methods (in the case of colistin) were done according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020). *Escherichia coli* ATCC 25922 was used as a control strain. The antimicrobials tested by disk diffusion were: amoxicillin/clavulanate (20/10 μg), ampicillin (10 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), fosfomycin (200 μg/50 μg of glucose-6-phosphate), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg) (Becton Dickinson). Extended-spectrum beta-lactamases were screened by the ESBL test following the CLSI guidelines (CLSI, 2020). Isolates were classified as susceptible, resistant to 1 or 2 antimicrobial categories, multidrug-resistant (MDR) if resistant to at least one agent in $\geq$ 3 antimicrobial categories; or extensively drug-resistant (XDR), if resistant to at least one agent in all but two or fewer antimicrobial categories (Magiorakos et al., 2012).

Identification of Antimicrobial Resistance Genes

Polymerase chain reaction (PCR) assays were performed using the supernatant of a boiled cell suspension of each isolate as DNA template. β-lactamase-encoding genes bla_{SHV-1}, bla_{TEM-1} and the five major groups bla_{CTX-M}: $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-25}$ were detected, as well as genes conferring resistance to sulfonamides (*sul1* and *sul2*), quinolones [*qnrB* and *aac(6')-lb-cr*] and colistin (*mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5*). Previously characterized strains from our laboratory collection were used as positive controls of the different genes in the corresponding PCR experiments. Water was used as negative control. The PCR products from the strains were sequenced (Genewiz). The obtained sequences were compared with those of the corresponding genes available in the GenBank. Primer sequences (Condalab, Spain) used in the study of antimicrobial resistance genes are listed in **Table 1**.

Virulence Determinants Detection

Virulence factor genes encoding for adhesins (*fimH-1, sfa, papA, pgaA*), siderophores (*iroN, iutA, irp-1, iucA*) and toxins (*hlyA, cnf-1, clbA clbB, clbN* and *clbQ*) were detected by PCR. To determine the presence of the complete *pks* genomic island, primers for the four most representative genes were used: *clbA* and *clbQ* as flanking primers, and *clbB* and *clbN* as internal primers (Johnson et al., 2008; Dubois et al., 2010; Suresh et al., 2018). The primer sequences (Condalab, Spain) used for the detection of the different VFGs are listed in **Table 2**. Previously characterized strains carrying the different VFGs were used as positive controls. Water was used as negative control.

Siderophore Assay

Siderophores production was determined according to the protocol described by Schwyn and Neiland (Schwyn and Neilands, 1987). Briefly, bacterial strains were cultured on chrome azurol S (CAS) (VWR) and hexadecyltrimethylammonium bromide (HDTMA) plates (Fisher Scientific). If a bacterium excretes a siderophore that removes iron from the dye complex, the color of the agar changes from blue to orange. *Acinetobacter baumannii* ATCC19606 was used as positive control.

Phylogroup Assignment Method

Escherichia coli strains were classified into seven phylogroups (A, B1, B2, C, D, E, and F) according to the PCR method designed by Clermont et al. (2013). Strains belonging to our group and whose phylogroup was previously identified were used as controls.

Typing of Colibactin-Positive Strains by Pulsed-Field Gel Electrophoresis

Due to the high prevalence of colibactin among the studied strains, we decided to analyze their clonal relationship by pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested DNA, following the protocol described by Durmaz et al. (2009). The profiles obtained were compared using the InfoQuestTM FPv.5.4 software (Bio-Rad Laboratories) and the unweighted pair group method with arithmetic mean to create dendrograms based on Dice's similarity coefficient. Isolates were clustered together if their similarity index was $\geq$ 85%.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows software, version 21.0. The Chi-square test was used to evaluate correlations among variables. p-values $<$ 0.05 were considered statistically significant.

RESULTS

Biofilm Formation and Quantification

Escherichia coli strains were analyzed for biofilm formation using M63 broth. Fifty-nine strains (15.7%) were classified as non-biofilm-forming isolates, 87 (23.1%) were classified as weakly biofilm-forming, 134 (35.6%) as moderately biofilm-forming, and 96 (25.6%) as strongly biofilm-forming strains.

Furthermore, the ability to form biofilm was investigated according to the origin of the isolates (urine, blood or respiratory tract) as shown in **Figure 1**; however, no correlation between strain source and biofilm formation ability was found ($p > 0.05$).

Antimicrobial Susceptibility Testing

The percentages of antimicrobial resistance are shown in **Figure 2**. Overall, the highest percentages of antibiotic resistance

TABLE 1 | Primers to detect antimicrobial resistance genes.

Target gene	Primer sequence (5′ → 3′)	Melting Temperature (Tm °C)	Product size (bp)	References
bla_{TEM-1} – F	TCGCCGCATACACTATTCTCAGAATGA	53	445	Monstein et al., 2007
bla_{TEM-1} – R	ACGCTCACCGGCTCCAGATTTAT			
bla_{SHV-1} – F	ATGCGTTATATTCGCCTGTG	49	747	Wiegand et al., 2007
bla_{SHV-1} – R	TGCTTTGTTATTCGGGCCAA			
$bla_{CTX-M-1}$ – F	AAAAATCACTGCGCCAGTTC	52	415	Woodford et al., 2006
$bla_{CTX-M-1}$ – R	AGCTTATTCATCGCCACGTT			
$bla_{CTX-M-2}$ – F	CGACGCTACCCCTGCTATT	52	552	Woodford et al., 2006
$bla_{CTX-M-2}$- R	CCAGCGTCAGATTTTTCAGG			
$bla_{CTX-M-9}$ – F	CAAAGAGAGTGCAACGGATG	52	205	Woodford et al., 2006
$bla_{CTX-M-9}$ – R	ATTGGAAAGCGTTCATCACC			
$bla_{CTX-M-8}$ – F	TCGCGTTAAGCGGATGATGC	52	666	Woodford et al., 2006
$bla_{CTX-M-8}$ – R	AACCCACGATGTGGGTAG			
$bla_{CTX-M-25}$ – F	GCACGATGACATTCGGG	52	327	Woodford et al., 2006
$bla_{CTX-M-25}$- R	AACCCACGATGTGGGTAG			
$bla_{CTX-M-15/28}$ – F	ATAAAACCGGCAGCGGTG	55	483	Leflon-Guibout et al., 2004
$bla_{CTX-M-15/28}$- R	GAATTTTGACGATCGGGG			
$bla_{CTX-M-14/27}$ – F	CGCTTTATGCGCAGACGA	57	785	Pai et al., 2001
$bla_{CTX-M-14/27}$- R	GATTCTCGCCGCTGAAGC			
sul1 – F	CTTCGATGAGAGCCGGCGGC	63	436	Guerra et al., 2004
sul1 – R	GCAAGGCGGAAACCCGCGCC			
sul2 – F	TCAACATAACCTCGGACAGT	55	707	Guerra et al., 2004
sul2 – R	GATGAAGTCAGCTCCACCT			
aac(6′)-Ib – cr –F	TTGCGATGCTCTATGAGTGGCTA	60	482	Park et al., 2006
aac(6′)-Ib – cr – R	CTCGAATGCCTGGCGTGTTT			
qnrB – F	GATCGTGAAAGCCAGAAAGG	52	469	Robicsek et al., 2006
qnrB – R	ACGATGCCTGGTAGTTGTCC			
mcr-1 – F	ATGCCAGTTTCTTTCGCGTG	59	502	Lescat et al., 2018
mcr-1 – R	TCGGCAAATTGCGCTTTTGGC			
mcr-2 – F	GATGGCGGTCTATCCTGTAT	59	379	Lescat et al., 2018
mcr-2 – R	AAGGCTGACACCCCATGTCAT			
mcr-3 – F	ACCAGTAAATCTGGTGGCGT	59	296	Lescat et al., 2018
mcr-3 – R	AGGACAACCTCGTCATAGCA			
mcr-4 – F	TTGCAGACGCCCATGGAATA	59	207	Lescat et al., 2018
mcr-4 – R	GCCGCATGAGCTAGTATCGT			
mcr-5 – F	GGACGCGACTCCCTAACTTC	59	608	Lescat et al., 2018
mcr-5 – R	ACAACCAGTACGAGAGCACG			

were observed with ciprofloxacin (48.7%), trimethoprim-sulfamethoxazole (47.9%), and ampicillin (38%). Only two strains were resistant to colistin (0.5%), and one to fosfomycin (0.3%). None was resistant to carbapenems (imipenem or meropenem). To determine whether biofilm formation correlates with antimicrobial resistance, the different categories of biofilm formation were compared with resistance profiles as shown in **Figure 3**. Non-biofilm-forming strains showed higher percentages of resistance to ciprofloxacin (74.6%) compared to biofilm-forming strains (**Figure 3H**). Thus, the higher the resistance to ciprofloxacin, the lower the biofilm production ability ($p < 0.0001$).

Regarding the antimicrobial resistance classification, 91 strains (24.2%) were susceptible to all the antimicrobial categories tested, 142 (37.8%) were resistant to one or two antimicrobial categories, 143 (38%) isolates were MDR, and none was XDR. The percentages for biofilm formation by antimicrobial resistance classification are shown in **Figure 4**. A direct relationship between antimicrobial susceptibility and ability to form biofilm was observed. However, no statistical relationship was found between biofilm formation ability and the antimicrobial resistance classification ($p = 0.053$).

Identification of Antimicrobial Resistance Genes

In total, 73 (19.4%) isolates were found to be ESBL-producing strains. The most common β-lactamase-encoding gene was $bla_{CTX-M-15}$ (n = 43, 58.9%), followed by bla_{TEM-1} (n = 33, 45.2%), $bla_{CTX-M-14}$ (n = 8, 12.3%), bla_{SHV-1} (n = 7, 9.6%), $bla_{CTX-M-27}$ (n = 5, 6.8%) and $bla_{CTX-M-28}$ (n = 3, 4.1%). None carried the $bla_{CTX-M-2}$, $bla_{CTX-M-8}$ or $bla_{CTX-M-25}$ genes, but 27 strains (37%) harbored more than one β-lactamase-encoding gene.

TABLE 2 | Primers to detect virulence factor genes.

Target gene	Primer sequence (5′ → 3′)	Melting temperature (Tm °C)	Product size (bp)	Function	References
iroN – F	AAGTCAAAGCAGGGGTTGCCCG	56	827	Siderophore uptake transmembrane transporter activity	Johnson et al., 2000
iroN – R	GACGCCGACATTAAGACGCAG				
irp1 – F	GGCGTCTCCTCCTTTGGTATT	60	1729	Gene encoding for an iron regulatory protein	Xu et al., 2000
irp1 – R	GTGATTCCCGCTGTTGATGTT				
iucA – F	AGTCTGCATCTTAACCTTCA	56	1100	Gene encoding for an aerobactin	Guerrieri et al., 2019
iucA – R	CTCGTTATGATCGTTCAGAT				
papA – F	ATGGCAGTGGTGTCTTTTGGTG	62	717	Fimbrial major pilin protein precursor	Johnson and Stell, 2000
papA – R	CGTCCCACCATACGTGCTCTTC				
fimH – F	CAGCGATGATTTCCAGTTTGTGTG	59	461	Type 1 fimbrin D-mannose specific adhesin precursor	Sáez-López et al., 2017
fimH – R	TGCGTACCAGCATTAGCAATGTCC				
sfa – F	CTCCGGAGAACTGGGTGCATCTTAC	65	410	S fimbriae	Houdouin et al., 2006
sfa – R	CGGAGGAGTAATTACAAACCTGGCA				
cnf-1 – F	AAGATGGAGTTTCCTATGCAGGAG	56	498	Cytotoxic necrotizing factor	Takahashi et al., 2006
cnf-1 – R	CATTCAGAGTCCTGCCCTCATTATT				
hlyA – F	AACAAGGATAAGCACTGTTCTGGC	59	1177	Hemolysin	Johnson and Stell, 2000
hlyA – R	ACCATATAAGCGGTCATTCCCGTCA				
iutA – F	GGCTGGACATCATGGGAACTGG	60	300	Ferric aerobactin receptor precursor	Johnson and Stell, 2000
iutA – R	CGTCGGGAACGGGTAGAATCG				
pgaA – F	GGCTTTGAAACTTCTTACTGC	60	209	Poly-beta-1,6-N-acetyl-D-glucosamine export protein	Shrestha et al., 2019
pgaA – R	CCTGTTTATCTTGCCCGGCC				
clbQ – F	CTTGTATAGTTACACAACTATTTC	54	821	Colibactin biosynthesis thioesterase ClbQ	Morgan et al., 2019
clbQ – R	TTATCCTGTTAGCTTTCGTTC				
clbA – F	CTAGATTATCCGTGGCGATTC	54	1002	Colibactin biosynthesis phosphopantetheinyl transferase ClbA	Morgan et al., 2019
clbA – R	CAGATACACAGATACCATTCA				
clbB – F	GATTTGGATACTGGCGATAACCG	54	579	Colibactin hybrid non-ribosomal peptide synthetase/type I polyketide synthase ClbB	Johnson et al., 2008
clbB – R	CCATTTCCCGTTTGAGCACAC				
clbN – F	GTTTTGCTCGCCAGATAGTCATTC	54	733	Colibactin non-ribosomal peptide synthetase ClbN	Johnson et al., 2008
clbN – R	CAGTTCGGGTATGTGTGGAAGG				

Regarding biofilm formation, 17 of the ESBLs-producing strains (23.3%) were non-biofilm-formers, 15 (20.5%) were weak biofilm-formers, 25 (34.2%) were moderate biofilm-formers, and 16 (21.9%) were strong biofilm-formers. However, no relationship was found between ESBL production and biofilm formation.

Of the 190 strains resistant to trimethoprim-sulfamethoxazole, 78 (41.1%), 131 (68.9%), and 47 strains (24.7%) harbored *sul1*, *sul2*, or both genes, respectively.

Among the 188 quinolone-resistant strains, 6 (3.2%) *qnrB* and 40 (21.3%) *aac (6')-Ib-cr* genes were detected. We observed a higher prevalence of the *aac (6')-Ib-cr* gene among the non-biofilm-forming strains, being the correlation statistically significant ($p = 0.008$). We detected two colistin-resistant strains, one of which carried the *mcr-1* gene.

Virulence Determinants Detection

The results about the prevalence of the different VFGs tested are shown in **Figure 5**. It was observed that the prevalence of siderophore-related genes was variable: *irp1* (77.1%), *iutA* (66.8%), *iucA* (52.7%), and *iroN* (45.5%). A significant correlation was found between biofilm formation ability and the presence of

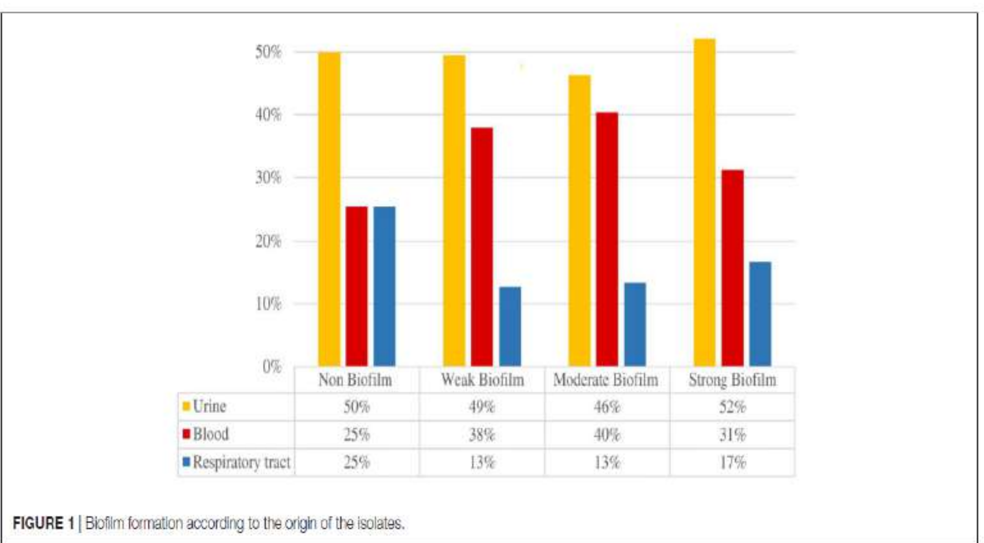

FIGURE 1 | Biofilm formation according to the origin of the isolates.

FIGURE 2 | Overall antimicrobial resistance rates expressed in percentage (%). AMC: Amoxicillin/clavulanic acid, AMP: Ampicillin, ATM: Aztreonam, CLO: Chloramphenicol, CAZ: Ceftazidime, CIP: Ciprofloxacin, CST: Colistin, CTX: Cefotaxime, FEP: Cefepime, FOS: Fosfomycin, GEN: Gentamicin, IMI: Imipenem, MER: Meropenem, SXT: Trimethoprim-sulfamethoxazole.

iroN ($p < 0.001$) or *iutA* ($p = 0.010$) genes. However, *iutA* gene was more prevalent among non-biofilm-forming strains.

The adhesin-encoding genes *fimH* and *pgaA* were the most frequent genes among the strains, with a prevalence of 92% and 84.6%, respectively. The prevalence of the other adhesin-encoding genes, such as *papA* and *sfa*, was 43.1% and 21.8%, respectively. A statistically significant correlation was found between biofilm formation and the presence of the *papA*, *fimH*, and *sfa* genes ($p < 0.001$).

Among the genes encoding toxins, *hlyA* and *cnf-1* showed a prevalence of 25% and 24.7%, respectively. The *clbA*, *clbB*, and *clbN* genes were present in 19.9% of the strains. *clbQ* gene was observed in 20.5% of the isolates. It is of note that a statistically significant correlation was confirmed between the presence of toxin-encoding genes and the ability to form biofilm: *hlyA* ($p = 0.0002$), *cnf* ($p < 0.001$), and colibactin-encoding genes (*clbA*, *clbB*, *clbN* and *clbQ*) ($p < 0.001$).

Siderophore Assay (Chrome Azurol S Test)

The 376 isolates were screened on CAS agar plates, a useful method for identifying siderophores in *E. coli* isolates and other Gram-negative bacteria. 199 (52.9%) siderophore-producing

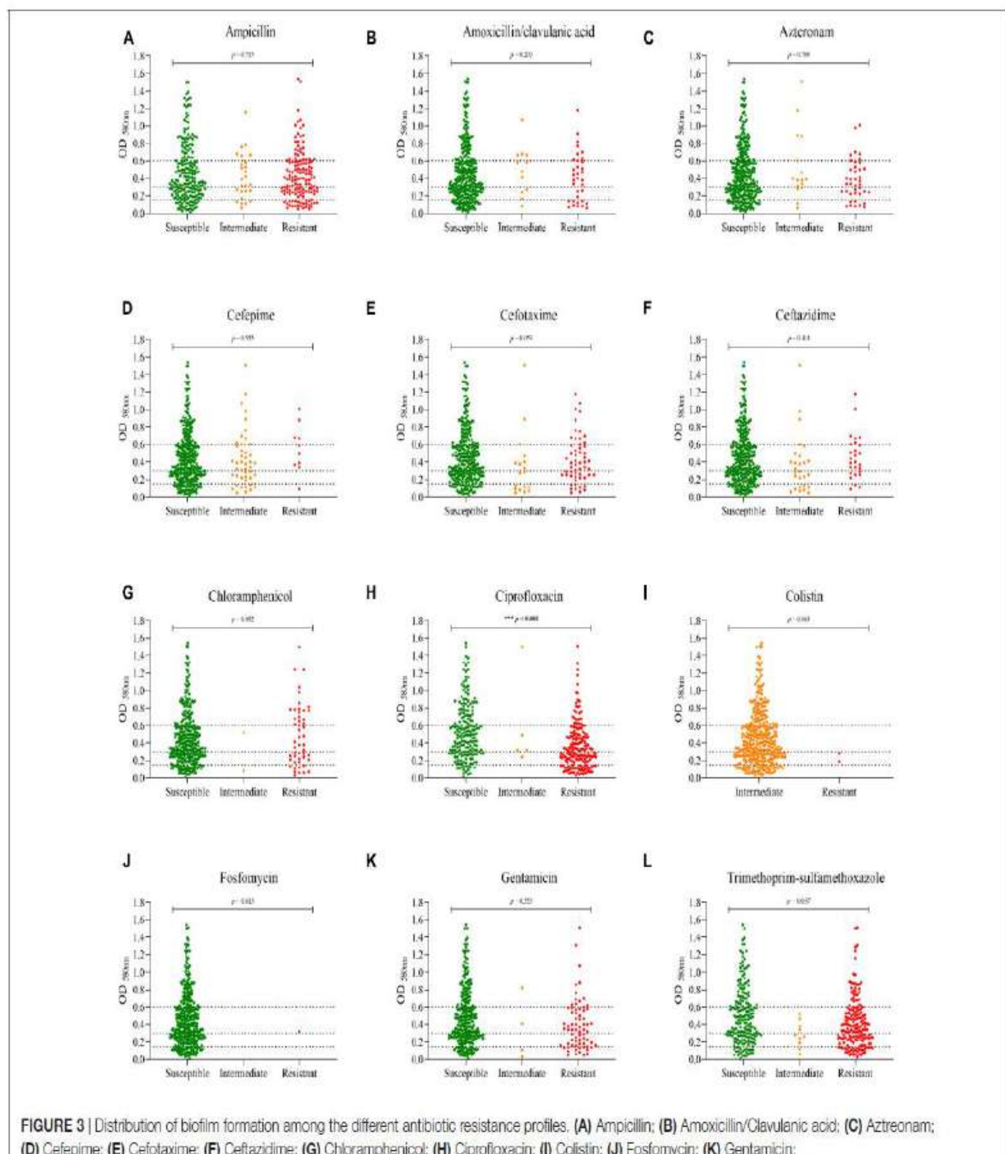

FIGURE 3 | Distribution of biofilm formation among the different antibiotic resistance profiles. **(A)** Ampicillin; **(B)** Amoxicillin/Clavulanic acid; **(C)** Aztreonam; **(D)** Cefepime; **(E)** Cefotaxime; **(F)** Ceftazidime; **(G)** Chloramphenicol; **(H)** Ciprofloxacin; **(I)** Colistin; **(J)** Fosfomycin; **(K)** Gentamicin; **(L)** Trimethoprim-sulfamethoxazole. The dotted lines indicate the cut-off points for each biofilm category [non-biofilm formers (OD $\leq$ 0.150), weak biofilm formers ($\geq$ 0.151 OD $\leq$ 0.300), moderate biofilm formers ($\geq$ 0.301 OD $\leq$ 0.60), or strong biofilm formers (OD $\geq$ 0.601)].

strains were found, as shown in **Figure 5**, but statistical analysis showed no relationship between CAS test positivity and biofilm formation ($p = 0.132$).

On the other hand, a statistically significant association between the positive CAS method and the presence of different siderophore-encoding genes *irp1*, *iucA*, *iutA* ($p < 0.001$) was found. The presence of the fimbriae H coding gene (*fimH*) was also correlated with the CAS test ($p = 0.025$). Contrarily, an association between the CAS test and the absence of the *sfa* gene ($p = 0.024$) was observed.

It is to note that strains resistant to antibiotics such as gentamicin ($p = 0.032$), ampicillin ($p = 0.001$), cefotaxime ($p = 0.001$), ceftazidime ($p = 0.002$), cefepime ($p = 0.001$) or aztreonam ($p < 0.001$) showed high siderophore production (positive CAS test). Likewise, ESBL-producing strains were statistically associated with a positive CAS test ($p = 0.001$). Finally, strains belonged to phylogroup B2 produced more siderophores than strains belonging to the other phylogroups ($p < 0.001$).

Phylogroup Assignment Method

In our study, the phylogenetic groups considered more virulent B2 and D, accounted for 72.9% of the *E. coli* isolates [B2: $n = 235$ (62.5%); D: $n = 39$ (10.4%)]. The less virulent groups were found in varying percentages [A: $n = 18$ (4.8%); B1: $n = 31$ (8.2%);

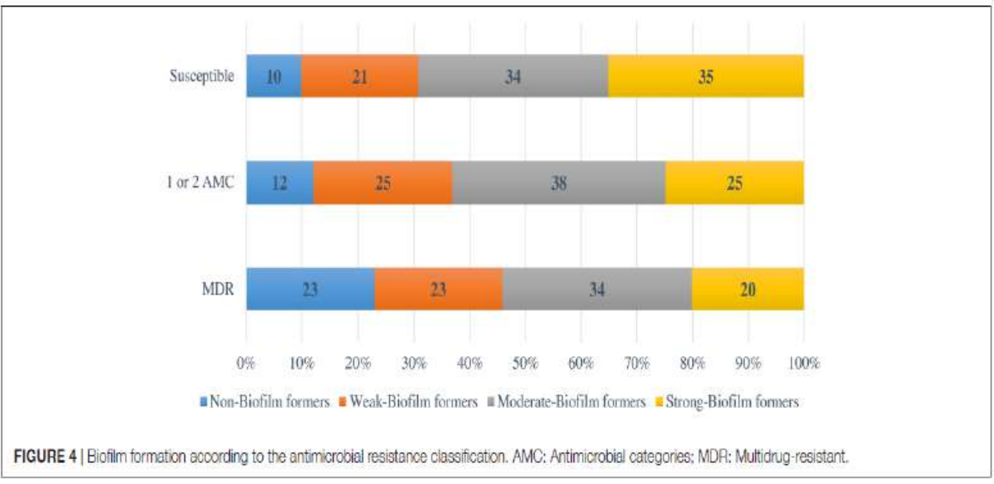

FIGURE 4 | Biofilm formation according to the antimicrobial resistance classification. AMC: Antimicrobial categories; MDR: Multidrug-resistant.

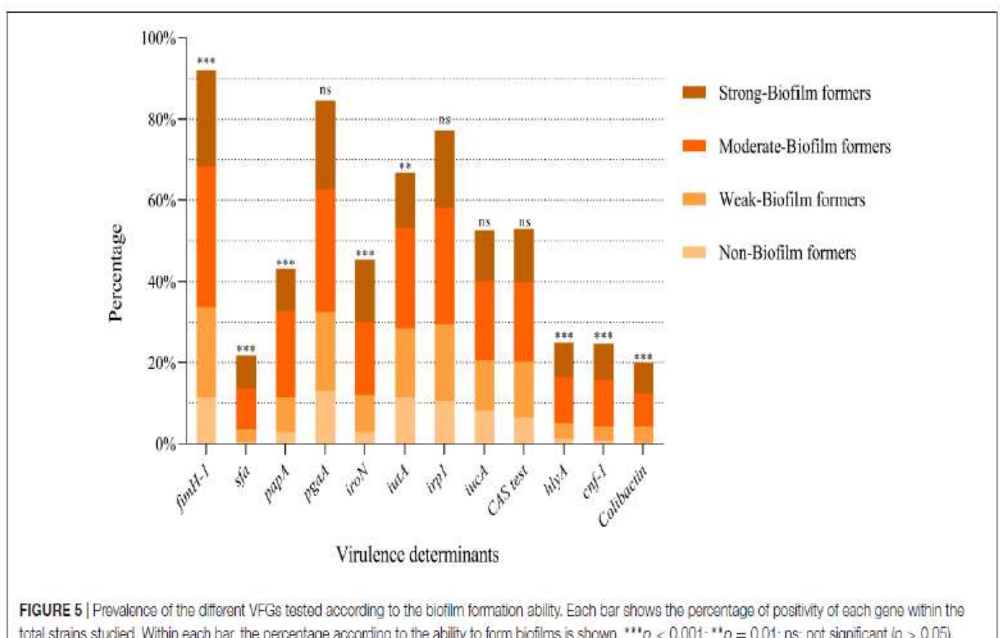

FIGURE 5 | Prevalence of the different VFGs tested according to the biofilm formation ability. Each bar shows the percentage of positivity of each gene within the total strains studied. Within each bar, the percentage according to the ability to form biofilms is shown. $^{***}p < 0.001$; $^{**}p = 0.01$; ns: not significant ($p > 0.05$).

C: $n = 18$ (4.8%); E: $n = 4$ (1.1%); F: $n = 26$ (6.9%); unknown: $n = 5$ (1.3%)].

According to biofilm classification, a great variety of phylogroups was observed in the non-biofilm-forming strains group. In contrast, a direct relationship between the ability to form biofilm and phylogroup B2 was observed, being this relationship statistically significant ($p < 0.001$) (**Figure 6**).

Characterization of Colibactin-Encoding *Escherichia coli* Strains

The presence of *clbA*, *clbB*, *clbN*, and *clbQ* genes confirmed the full presence of the *pks*-island in 75 strains. Among these, 74 presented the ability to form biofilm. We compared whether the presence of colibactin-encoding genes was related to biofilm formation ability and we found a statistically significant relationship between these two variables ($p < 0.001$).

As shown in **Table 3**, colibactin-producing strains showed a high prevalence of most of the VFGs tested (*iroN*, *irp1*, *papA*, *fimH*, *cnf*, *hlyA*, *pgaA*, and *sfa*) ($p < 0.05$), but had a low prevalence of the *iucA* and *iutA* genes. The relationship between susceptibility to most of the antimicrobials tested (amoxicillin/clavulanic acid, ampicillin, aztreonam, cefepime, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole) and the presence of colibactin genes was statistically significant ($p < 0.05$). Only two of the colibactin-encoding strains were ESBL-producers, showing an inverse correlation between colibactin genes and ESBL production ($p < 0.001$). Most colibactin-positive strains were isolated from urine ($n = 45$;

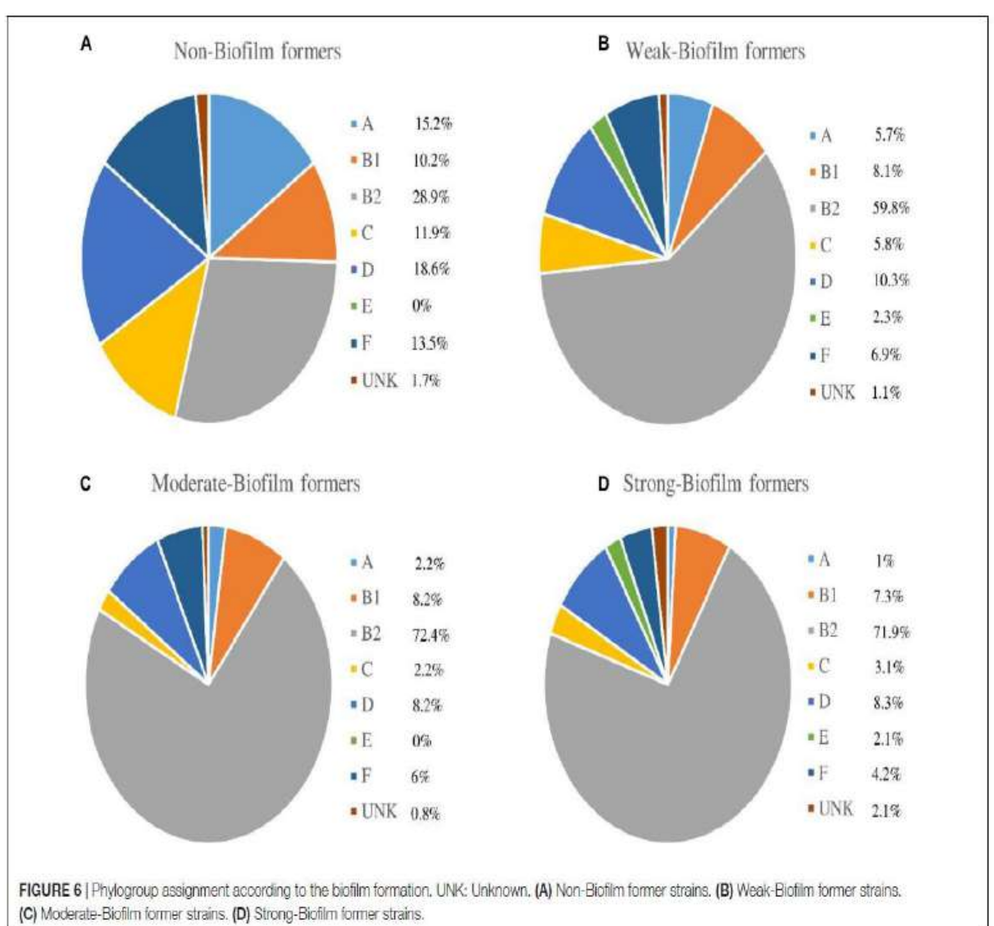

FIGURE 6 | Phylogroup assignment according to the biofilm formation. UNK: Unknown. **(A)** Non-Biofilm former strains. **(B)** Weak-Biofilm former strains. **(C)** Moderate-Biofilm former strains. **(D)** Strong-Biofilm former strains.

60%), followed by strains from blood (n = 22; 29.3%), and the respiratory tract (n = 8; 10.7%), but no correlation was found between the strain source and the presence of colibactin-encoding genes.

The majority of the colibactin-positive strains belonged to phylogroup B2, and the analysis of the clonal relatedness by PFGE of the colibactin-positive *E. coli* strains showed 48 different clusters with a Dice similarity index ≥ 85%. This indicates that the *E. coli* isolates harboring the colibactin toxin exhibit a high clonal diversity (**Figure 7**).

DISCUSSION

Extraintestinal pathogenic *E. coli* strains are a group of bacteria that can cause urinary tract, bloodstream and other non-intestinal infections, both in healthcare settings and in the community (Manges et al., 2019). Several studies have investigated the association between antimicrobial resistance and/or the presence of some VFGs and the ability to form biofilms (May and Okabe, 2011; Friedlander et al., 2015; Cepas et al., 2019). However, the virulence genes described in recent years need to be studied in more detail, as they may be related to biofilm formation. In this study, we characterized 376 strains to investigate the possible relationship between antimicrobial resistance and/or the presence of selected virulence determinants and the ability to form biofilms. Our results showed that almost all the isolates studied (84.3%) were able to form biofilms, and this high rate might be related to some of the genes and features observed among the strains studied.

In terms of antimicrobial resistance, we found high rates of resistance to ciprofloxacin (48.7%), trimethoprim-sulfamethoxazole (47.9%), and ampicillin (38%), and fully susceptible strains to carbapenems (imipenem and meropenem). Comparing our results with the SMART study (Cantón et al., 2021), which investigated Gram-negative bacilli isolated from intra-abdominal, urinary, bloodstream and lower respiratory tract infection from 2016 to 2018 in 11 participating Spanish Hospitals, we found similar resistance rates for cephalosporines, carbapenems and colistin but their antimicrobial resistance rates to ciprofloxacin were lower (32.3%). Besides, they found a lower rate of ESBL-producing strains (8.6%). According to the data from the 2017 European Antimicrobial Resistance Surveillance Network report (European Centre for Disease Prevention and Control [ECDC], 2017), which is based on antimicrobial resistance data from invasive isolates, we

TABLE 3 | Characterization of colibactin-encoding *Escherichia coli* strains.

Strain	Origin	Phylogroup	Biofilm	Adhesines				Siderophores					Toxins				ARGs			
				fimH	*sfa*	*papA*	*pgaA*	*iroN*	*iutA*	*irp1*	*iucA*	CAS test	*hlyA*	*cnf1*	*clbA*	*clbQ*	$bla_{ctx-m-15}$	$bla_{ctx-m-14}$	*sul1*	*sul2*
HUB561	Urine	B2	Moderate	+			+		+	+	+	+			+	+				+
HUB562	Urine	B2	Moderate	+	+	+	+	+		+			+	+	+	+				
HUB564	Urine	B2	Weak	+	+	+	+	+		+		+	+	+	+	+				
HUB569	Urine	B2	Strong	+	+	+	+	+		+		+	+	+	+	+				
HCB0012	Blood	B2	Moderate	+	+	+	+	+		+		+	+	+	+	+				
HMT142	Blood	B2	Strong	+	+		+	+		+		+	+	+	+	+				
HCB0015	Blood	B2	Strong	+	+	+	+	+		+		+	+	+	+	+				
HMT0019	Urine	B2	Moderate	+	+	+	+	+		+		+	+	+	+	+			+	+
HCB0029	Blood	B2	Moderate	+		+	+			+		+			+	+				
HCB0031	Blood	B2	Moderate	+	+	+	+	+		+			+	+	+	+				
HCB0035	Blood	B2	Strong	+	+	+	+	+		+		+			+	+				
HCB881	Blood	B2	Strong	+	+	+	+	+		+			+	+	+	+				
HCB0038	Blood	B2	Weak	+	+	+	+	+		+		+	+	+	+	+	+			
HCB0039	Blood	B2	Moderate	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+
HCB0042	Urine	B2	Strong	+			+			+		+			+	+			+	+
HCB0044	Urine	B2	Moderate	+	+	+	+	+	+	+	+	+	+	+	+	+				
HCB0045	Urine	B2	Moderate	+	+	+	+	+	+	+	+	+	+	+	+	+				+
HCB0047	Urine	B2	Strong	+	+	+	+	+	+	+	+	+	+	+	+	+				+
HCB883	Urine	B2	Strong	+	+		+	+		+		+	+	+	+	+				
HCB196	Blood	B2	Weak	+	+		+	+	+	+	+	+	+	+	+	+				
HCB0113	Respiratory	B2	Moderate	+	+	+	+	+		+			+	+	+	+				
HCB197	Respiratory	B2	Strong	+	+		+	+		+			+	+	+	+				
HCB198	Respiratory	B2	Strong	+	+		+	+		+			+	+	+	+				
HCB889	Urine	B2	Moderate	+	+	+	+	+		+			+	+	+	+				
HCB143	Urine	B2	Strong	+	+	+	+	+	+	+	+	+	+	+	+	+				
HCB150	Urine	B2	Strong	+	+	+	+	+	+	+	+	+	+	+	+	+				
HCB154	Urine	B2	Weak	+		+	+		+	+	+	+	+	+	+	+				
HUB576	Urine	B2	Strong	+	+	+	+	+		+			+	+	+	+				
HUB582	Blood	B2	Moderate	+		+	+			+			+	+	+	+				
HMT372	Urine	B2	Moderate	+	+	+	+		+	+	+	+	+	+	+	+				+
HMT378	Urine	B2	Moderate	+	+	+	+	+		+			+	+	+	+				
HMT379	Blood	B2	Moderate	+	+	+	+	+	+	+		+	+	+	+	+				
HMT385	Urine	B2	Weak	+			+		+						+	+				+
HMT386	Urine	B2	Strong	+	+	+	+	+		+			+	+	+	+				
HUB605	Blood	B2	Strong	+	+	+	+	+	+	+	+	+	+	+	+	+				
HMT389	Urine	B2	Weak	+	+	+	+	+	+	+	+		+	+	+	+				
HMT396	Urine	B2	Moderate	+	+	+	+	+	+	+		+	+	+	+	+				
HMT399	Urine	B2	Moderate	+	+	+	+	+		+			+	+	+	+				
HUB610	Blood	B2	Weak	+		+	+	+	+	+	+	+	+		+	+				
HUB647	Urine	B2	Strong	+	+		+	+		+			+		+	+				
HCB207	Urine	B2	Strong	+	+		+	+		+			+	+	+	+			+	
HCB210	Blood	B2	Strong	+	+	+	+	+		+			+	+	+	+				
HCB214	Blood	B2	Strong	+	+		+	+		+			+	+	+	+				
HUB648	Urine	B2	Moderate	+	+	+	+	+	+	+		+	+	+	+	+				
HCB218	Urine	B2	Moderate	+			+			+					+	+				
HUB649	Urine	B2	Strong	+	+		+	+		+			+	+	+	+				
HUB650	Urine	B2	Strong	+		+	+		+	+	+				+	+				
HUB652	Urine	B2	Strong	+	+	+	+	+	+	+	+		+	+	+	+				
HUB653	Urine	B2	Moderate	+	+		+	+		+			+	+	+	+				
HUB655	Urine	B2	Strong	+		+	+	+	+	+	+	+		+	+	+				

(Continued)

TABLE 3 | (Continued)

Strain	Origin	Phylogroup	Biofilm	Adhesines				Siderophores					Toxins				ARGs			
				fimH	*sfa*	*papA*	*pgaA*	*iroN*	*iutA*	*irp1*	*iucA*	CAS test	*hlyA*	*cnf1*	*clbA*	*clbQ*	$bla_{CTX-M-15}$	$bla_{CTX-M-14}$	*sul1*	*sul2*
HUB665	Urine	B2	Moderate	■	■	■	■	■		■			■	■	■	■				
HUB666	Urine	B2	Moderate	■	■	■	■	■	■	■	■	■	■	■	■	■				
HCB221	Respiratory	B2	Moderate	■	■		■	■		■			■	■	■	■				
HCB224	Blood	B2	Strong	■	■	■	■	■		■			■	■	■	■				
HCB226	Blood	B2	Strong	■		■	■		■	■	■	■	■	■	■	■				
HCB227	Blood	B2	Moderate	■	■		■	■	■	■	■				■	■				
HCB231	Urine	UN	Weak	■		■	■			■					■	■				
HCB232	Urine	B2	Non producer	■	■	■	■	■	■	■	■		■	■	■	■			■	
HCB233	Urine	B2	Weak	■			■		■	■	■				■	■				
HMT400	Urine	B2	Weak	■	■	■	■	■		■			■	■	■	■				■
HMT410	Urine	B2	Moderate	■	■		■	■	■	■	■	■			■	■		■		
HMT415	Urine	B2	Strong	■	■	■	■	■		■	■		■	■	■	■				
HMT418	Blood	B2	Weak	■		■	■		■	■	■	■	■	■	■	■				
HMT426	Urine	B2	Moderate	■	■	■	■	■	■	■	■		■	■	■	■				■
HUB809	Respiratory	B2	Strong	■	■	■	■	■		■			■	■	■	■				■
HUB810	Respiratory	B2	Weak	■	■	■	■		■	■		■	■	■	■	■				
HCB477	Urine	B2	Moderate	■	■	■	■	■	■	■	■		■	■	■	■				
HCB476	Respiratory	B2	Moderate	■	■		■	■		■			■	■	■	■				
HCB475	Respiratory	B2	Weak	■	■	■	■	■	■	■	■	■	■	■	■	■				
HCB473	Urine	B2	Weak	■			■		■	■	■	■			■	■				
HCB469	Blood	B2	Moderate	■	■	■	■	■		■		■	■	■	■	■				
HCB463	Urine	B2	Strong	■	■		■	■		■			■	■	■	■				
HCB457	Urine	B2	Weak	■		■	■			■		■			■	■				
HCB458	Blood	B2	Moderate	■	■	■	■	■		■		■	■	■	■	■				
HCB455	Urine	B2	Moderate	■	■	■	■	■	■	■		■	■	■	■	■				

Colored squares denote the presence of the gene or positivity of the test.
ARGs: antimicrobial resistance genes. UN: Unknown.

found similar percentages of fully susceptible strains but a higher rate of MDR in the collection under study. They also reported the highest average resistance percentage for aminopenicillins (58.7%), followed by fluoroquinolones (25.7%). These differences could be probably due to most of the strains in the present study were isolated from urine, and some of the most common antibiotics prescribed in UTIs are ciprofloxacin and trimethoprim-sulfamethoxazole. Previous studies conducted by our group showed no statistically significant relationship between biofilm formation ability and ciprofloxacin resistance among *E. coli* strains, but this association was found in *Pseudomonas aeruginosa* (Cepas et al., 2019). However, after analyzing whether this association existed in our study, a statistically significant association was found between non-biofilm-forming strains and resistance to ciprofloxacin. These variations among the data could be due to the greater number of strains analyzed in the present research. Several studies reported that quinolone-resistant strains were less virulent than quinolone-susceptible strains, observing that fimbriae genes, associated with biofilm formation, were less prevalent among nalidixic acid-resistant *E. coli* isolates (Vila et al., 2002; Horcajada et al., 2005). Moreover, the study by Fàbrega et al. (2014) showed that the acquisition of quinolone resistance was related to a decrease in biofilm formation in *Salmonella enterica* strains (Fàbrega et al., 2014) which may explain our results.

Regarding ESBL, these were highly prevalent in our study (19.4%) compared to the study conducted by Flament-Simon et al. (2020) in which only 13 of the strains (6.6%) isolated in Spain and France in 2016 produced ESBL enzymes (Flament-Simon et al., 2020). In agreement with our results, several epidemiological studies confirm that $bla_{CTX-M-15}$ is one the most common ESBL among clinical isolates from Spain. However, $bla_{CTX-M-14}$ and $bla_{CTX-M-27}$ have also been found in this country (Dahbi et al., 2014; Merino et al., 2016, 2018). Interestingly, three strains carrying $bla_{CTX-M-28}$ were found in our study, which is less prevalent than other $bla_{CTX-M-1}$ enzymes (Livermore et al., 2007). When we analyzed the relation between biofilm formation and ESBL production, we did not find any correlation between these two variables. However, Shrestha et al. (2019) found a positive correlation between these two features (Shrestha et al., 2019). The differences between the two studies could be due to the higher percentage of beta-lactamases (50.9% vs. 19.4%) they

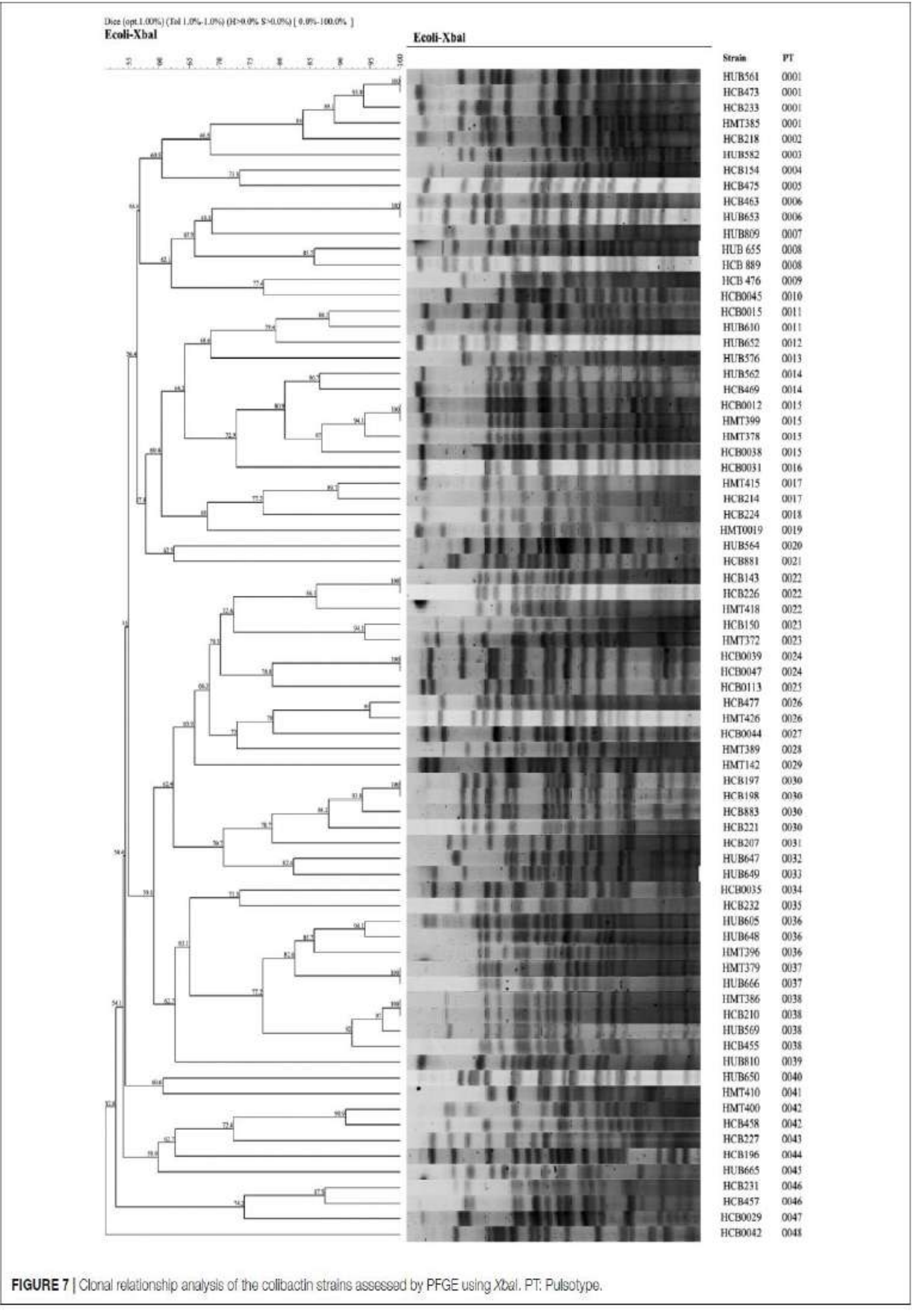

FIGURE 7 | Clonal relationship analysis of the colibactin strains assessed by PFGE using *Xba*I. PT: Pulsotype.

found. These results showing a worrying situation, since having a high percentage of ESBL among biofilm-forming strains makes very difficult their eradication.

As for virulence, we found 14 (3.7%) strains carrying all the VFGs tested. However, most were associated with low antimicrobial resistance rates. We observed a statistically significant correlation between the presence of different siderophores, adhesins or toxins and the ability to form biofilms. Siderophores are small molecules with high affinity for iron (Holden and Bachman, 2015). They are considered an important virulence factor in most Gram-negative bacteria. In *S. maltophilia*, iron plays a significant role in biofilm formation

and production of EPS (Kalidasan et al., 2018). In *P. aeruginosa*, siderophore-deficient clones showed reduced biofilm formation ability (Banin et al., 2005). In *E. coli*, studies by May and Okabe (2011) have shown that biofilm formation is favored in media with low iron concentrations (May and Okabe, 2011). In support of previous studies, we found a significant correlation between the presence of siderophore-encoding genes, such as *iroN*, and the ability to form biofilms. In addition, resistant strains produced more siderophores than their susceptible counterparts. Recently, it has been reported that the introduction of the mobile genetic element ICEKp from *Klebsiella pneumoniae* or a plasmid encoding YbtPQ (a siderophore importer encoded in the yersiniabactin cluster) reduced the susceptibility of *E. coli* to a wide range of antimicrobials (Farzand et al., 2021). Regarding adhesins, type 1 fimbriae are the best known adhesive organelles among Enterobacteriaceae (Abdelhalim et al., 2020) playing an important role in the initial steps of biofilm formation. As expected, we found a significant association between the presence of the *fimH* gene and biofilm formation. The adhesins *sfa* and *papA* also showed a significant association with biofilm formation. Although poly-β-1,6-N-acetyl-D-glucosamine polymer is associated with biofilm formation by mediating cell-to-cell adhesion and attachment to surfaces (Sharma et al., 2016), our results showed no statistical correlation between the presence of the *pgaA* gene and the capacity to form biofilms.

An important finding of the present work is the high presence of the toxin colibactin among the strains under study. Colibactin is a virulence determinant and a genotoxic enzyme synthesized by polyketide synthases and encoded by a 54-kb genomic island designated *pks* (Faïs et al., 2018). Colibactin may induce DNA damage in the host and has been correlated with colorectal cancer in humans (Wernke et al., 2020). Previous studies have confirmed the presence of *pks*-positive strains among ExPEC (Auvray et al., 2021). Interestingly, 75 of our isolates (19.9%) were colibactin-encoding strains, a higher percentage than this observed by Suresh et al. (2018). Analysis of the association between colibactin and other VFGs showed a significant correlation of this toxin with the siderophore-encoding genes *iroN* and *irp1;* the adhesin-encoding genes *papA*, *fimH*, *pgaA*, and *sfa*; and the toxin genes *cnf-1* and *hlyA*. Iron is an essential element for the survival of *E. coli*. Previous studies confirm that the *pks* island is involved in the synthesis of siderophores such as yersiniabactin, enterobactin, and salmochelins via phosphopantetheinyl transferase ClbA (Martin et al., 2013). In addition, it has been suggested that *E. coli* strains carrying hemolysin and colibactin have advantages in colorectal colonization and subsequent cancer (Yoshikawa et al., 2020). As in our case, it has been previously reported that phylogenetic group B2 is predominant among colibactin-encoding *E. coli* strains (Sarshar et al., 2017). However, the high clonal diversity of PFGE analysis among colibactin-positive *E. coli* isolates studied suggests that colibactin may be distributed in a wide variety of strains and clones. Worryingly, Putze et al. (2009) detected colibactin-related genes associated with an ICE-like element in several enterobacteria, not only in *E. coli*, paving the way for the spread of this gene cluster among species (Putze et al., 2009). Furthermore, we observed low rates of resistance to antimicrobial agents among colibactin-positive isolates, which is consistent with previous studies (Sarshar et al., 2017; Morgan et al., 2019). Although colibactin-encoding genes may have a direct influence on other biosynthetic pathways, our findings support the hypothesis that the presence of colibactin-encoding genes may be related to biofilm formation. In this sense, several studies showed a high capacity for biofilm formation among the *pks*-carrying strains studied (Raisch et al., 2014; Suresh et al., 2018). Likewise, Dejea et al. (2014) conducted a study in two geographically distinct cohorts from the United States and Malaysia finding that 89% of cases of right-sided colorectal tumors presented biofilms in the biopsies samples, concluding that a significant association between the presence of biofilms and colorectal cancer exists (Dejea et al., 2014). In another study of patients with familial adenomatous polyposis, the bacterial biofilms found were composed predominantly by *E. coli* and *Bacteroides fragilis*. Genes encoding colibactin (*clbB*) were found at high levels in strains from these patients compared to strains isolated from healthy individuals (Dejea et al., 2018). Additionally, uropathogenic *E. coli* strains carrying the *pks* island have also been described. Chagneau et al. (2021) detected colibactin in 24.7% of urine samples from patients with community-acquired pyelonephritis, cystitis or asymptomatic bacteriuria (Chagneau et al., 2021). They also reported that colibactin was produced during UTIs and induced DNA damage in urothelial cells (Chagneau et al., 2021). Similarly, the study conducted by Morgan and collaborators in an *in vivo* model of ascending UTI in rats, showed that isolates carrying the *cnf-1*, *clbA* and *clbQ* genes induced severe UTIs within 48–72 h (Morgan et al., 2019). Thus, strains carrying colibactin-encoding genes can produce biofilms and cause severe disease not only in the intestine but also in the urinary tract, and probably in other less studied anatomic sites.

CONCLUSION

Ciprofloxacin resistance was associated with lower biofilm production ability among the *E. coli* strains under study. Furthermore, biofilm formation ability was correlated with the presence of some siderophores, adhesins, and toxins. The high percentage of strains harboring the colibactin-encoding genes and the significant correlation between this toxin and biofilm formation, suggest that colibactin could be a promising therapeutic target to prevent biofilm formation. Nevertheless, further studies are needed to confirm our hypothesis and to better understand the impact of colibactin production on human health.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SS and VB: conceptualization and writing—original draft preparation. VB, YG, CR, and MS: methodology. VB: statistical analysis. VB, CR, and SS: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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IX. Discussion

Biofilm research is a critical topic in the field of microbiology and infectious diseases. Biofilms are involved in crucial processes in nature, but they also have severe implications for human health. Therefore, biofilm research should be aimed at exploring the complexity of these microbial structures, the mechanisms involved in their formation, and new treatments to prevent and control them.

This thesis attempts to examine this health problem from different points of view: (i) the study of interactions between species within biofilms; (ii) the search for new genes involved in biofilm formation; (iii) the search for new molecules to treat biofilms; and (iv) the relationship between biofilms, virulence factors and antimicrobial resistance. Therefore, the discussion of this work is structured according to the specific objectives of the doctoral thesis.

Communication between species within biofilms can increase or decrease their biomass, depending on the specific interactions of synergy or antagonism that may exist between them. There is currently growing interest in understanding these complex relationships due to their impact on human health.

One of the polymicrobial biofilms that has attracted the most attention in research is that identified in the urinary catheters of patients with CAUTIs. It has been found that approximately 100 million urethral catheters are used annually worldwide, and 3-10% of catheterised patients present bacteriuria within 24 h of catheterisation, leading to between 9 and 27 million cases of CAUTIs per year worldwide (228).

In short-term catheterisation (up to 7 days), the catheter is usually colonised by a single species. However, if the catheter remains for a longer period of time, these infections are more likely to be polymicrobial (229). Several studies have shown that potentially pathogenic polymicrobial communities are observed in some CAUTIs through independent culture analysis of Foley catheters (46,230). In Bangladesh, a study conducted by Haque *et al.* found infections between *E. coli*/*Staphylococcus saprophyticus*, *E.*

coli/*Enterococcus* spp., *E. coli*/*Klebsiella* spp., and *E. coli*/*Proteus* spp. (231). Galván *et al.* also reported that some of the dual-species associations with higher prevalence in urine samples were *K. pneumoniae*/*E. coli*, *E. coli*/*E. faecalis*, *K. pneumoniae*/*E. faecalis*, and *K. pneumoniae*/*P. mirabilis*, accounting for 26%, 10%, 8.5%, and 7% of cases, respectively (232).

In **paper 1**, we focused on assessing interspecies interactions in polymicrobial biofilms of *K. pneumoniae* and *E. faecalis,* as this interaction has been little studied thus far. In this context, we found a dominance of *E. faecalis* over *K. pneumoniae,* supported by the competitive index (CI) and the quantification of viable cells, indicating an antagonistic relationship between the two species, and the inhibitory effect of *E. faecalis* against *K. pneumoniae.*

In an earlier study by Galván *et al.*, neither antagonistic nor cooperative interactions were observed between *K. pneumoniae* and *E. faecalis*. Nonetheless, neutral interactions were observed between these two species with similar numbers of viable cells when comparing mixed and single cultures (232). Our results are in contrast with this previous work, showing an advantage for *E. faecalis* in co-culture. These differences between the two studies are due to changes in methodology. Galvan *et al.* used an artificial urine medium without glucose. However, when it was tested by our group, sufficient growth of the *E. faecalis* strains was not allowed. Therefore, we used glucose-enriched trypticase soy broth (TSB) and glucose-enriched human urine and observed the same inhibitory effect in both cases. We also compared human urine without glucose, obtaining neutral interactions between the two species. This could suggest that a difference in the interactions between the two species could occur in diabetic and non-diabetic patients, although this hypothesis needs deeper study.

It is well known that bacteria produce different compounds that could interfere with the growth of other species, such as hydrogen peroxide, organic acids, or bacteriocins (233). We discarded bacteriocin production because we observed antibacterial and antibiofilm effects only with non-pH-adjusted lyophilised cell-free supernatants; thus, we focused on producing organic acids as the cause of *K. pneumoniae* inhibition.

In some polymicrobial biofilms, organic acids produced by one of the involved species cause a pH change in the surrounding medium that affects the other species growing in the biofilm (65). Therefore, Therefore, while the producer strain might benefit from its waste product itself or not be affected by it, it might accidentally interfere with the growth of the other species (Nadell *et al.* 2016). Thus, in our case, when lactic acid in the supernatants was measured, the concentration was sufficient to inhibit the growth and biofilm development of *K. pneumoniae*.

K. pneumoniae is one species that can produce urease after attachment to urinary catheters. Urease hydrolyses urea from urine, releases ammonia and CO_2, and increases the urine pH (234,235). However, we hypothesise that this fact was probably not sufficient to counterbalance the effect of the lactic acid produced by *E. faecalis*.

Lactic acid is the main product of *Enterococcus* fermentation under conditions of excess glucose and limited oxygen content. It is produced by the reduction of pyruvate for the regeneration of NAD^+ for ongoing glycolysis (236). We decided to use *E. faecalis* mutant strains defective in lactic acid production to confirm our hypothesis. The reduction in the colony number of *K. pneumoniae* was not statistically significant when *E. faecalis* V583 *Δldh-1* or *Δldh-1*/*Δldh-2* mutant strains were involved in the polymicrobial biofilm. Most lactic acid production is due to the *ldh-1* gene, while *ldh-2* plays a minor role in this process (237,238). This suggests that lactic acid production could give *E. faecalis* an advantage over *K. pneumoniae* or other species, as some *E. faecalis* strains are resistant to this acid and can adapt to different pH ranges growing under highly acidic conditions (239,240).

E. faecalis has been found to inhibit the growth of other bacteria in various environments. *Streptococcus gordonii* was entirely inhibited when co-cultured with *E. faecalis* in root canals (241). Another study showed that the presence of *E. faecalis* limits the growth of *Listeria monocytogenes* in polymicrobial biofilms at 39 °C due to competition for nutrients and the production of toxic metabolites (242). The metabolomics study conducted by Kart *et al.* showed that *E. faecalis* inhibits hyphal development and biofilm

formation of *C. albicans*. In addition, putrescine and pipecolic acid were detected in high concentrations in this polymicrobial biofilm, and the anti-QS activity was also higher in polymicrobial biofilms than in mono-species biofilms (243).

On the other hand, it has been found that *E. faecalis* can synergistically coexist with the uropathogen *P. mirabilis*. *E. faecalis* enhance the urease activity of *P. mirabilis* during *in vitro* urine co-culture. This fact could increase the severity of the disease and cause increased urine pH, crystalline deposits, catheter encrustation, and eventually, urolithiasis and bacteraemia (244). In another study using the same species, biofilm biomass increased due to secretion of EPS but no increase in bacterial cell count (245). However, we noted that although glucose-enriched TSB broth was used in this study, *E. faecalis* did not inhibit the growth of *P. mirabilis* by producing lactic acid, which is probably counteracted by the production of urease by this species.

Considering this effect of lactic acid, several researchers have explained how some lactic acid bacteria can be used as candidates for the development of probiotic microorganisms. Thus, probiotics have to meet specific requirements: (i) they should be isolated from the hosts for which they are intended to act; (ii) they should be able to survive in the intestinal tract; and (iii) they should produce bacteriostatic molecules (246).

Probiotics have been studied in adult females and children and used for urogenital tract health (247–249). *E. faecalis* has been proposed as an intestinal probiotic due to its adhesion to intestinal cells and strengthening of the epithelial barrier (250). Furthermore, *E. faecalis* (DSM 16431) is marketed under the brand name Symbioflor (SymbioPharm, Herborn, Germany) being not only recommended as an intestinal probiotic, but also suggested for the treatment of chronic recurrent hypertrophic sinusitis (251). Thus, *E. faecalis* could be considered a therapeutic or prophylactic probiotic strategy against urogenital infections, as they could produce lactic acid during adhesion to catheters, prevent adhesion of other bacteria, or reduce available nutrients through competition. All these findings lead us to continue the investigation of potential lactic acid bacteria as biocontrol agents for biofilm formation on

indwelling catheters in the context of UTIs, leading to new approaches to preventive measures.

Following the search for new strategies to control biofilms, we used the random transposon insertion technique in **paper 2** to find new genes involved in biofilm formation in *E. coli*, which could the focus for antibiofilm strategies. We created a random mutant library from which the 20 mutant strains with the lowest biofilm formation ability were analysed. Phenotypic characterisation of these strains was performed using swimming, congo red (CR), and haemagglutination assays. Finally, the mutant strains Tn463 and Tn263 were selected for whole-genome sequencing.

In the Tn463 strain, the transposon was inserted into the *csgA* and *cysB* genes. Because the *csgA* gene has previously been associated with biofilm formation, this mutant was discarded for the following analysis. The *csgA* gene encodes CsgA subunits, which are the primary component of curli amyloid fibres, essential in the adhesion phase (133).

In the Tn263 strain, the transposon was inserted into the *purL* gene, which encodes the 5′-phosphoribosyl-formyl-glycinamide amidotransferase involved in the *de novo* purine nucleotide biosynthetic pathway (252). Further analysis of Tn263, the knockout (*ΔpurL::cat*), and the complemented (*ΔpurL/purL+*) strains showed that *ΔpurL::cat*, like Tn263, also lost the ability to form biofilms and resulted in light-pink colonies associated with defective curli phenotypes on CR agar. The complemented *ΔpurL/purL+* strain showed absorbance values similar to those of the wild-type strain and restored the ability to form curli fimbriae and biofilms. Furthermore, the Tn263 and *ΔpurL::cat* strains regained their ability to form biofilms when inosine monophosphate (IMP) was added to the media. IMP is converted to guanosine 5′-monophosphate (GMP) or adenosine 5′-monophosphate (AMP) by various enzymes of the purine biosynthesis pathway (253).

These results demonstrate the crucial role of the *purL* gene and the purine biosynthesis pathway in curli production and, thus, in biofilm production. Garavaglia *et al.* found that, in *E. coli*, inactivation of the *purH*

gene, which is also involved in the purine pathway, resulted in a white phenotype on CR, and *csgDEFG* transcript levels decreased 7-fold. This suggests that since curli production and *csgDEFG* expression are dependent on c-di-GMP, changes in nucleotide biosynthetic pathways may affect c-di-GMP production (254).

Interestingly, another study showed that deletion of the *purF* gene in UPEC strains abolished *de novo* biosynthesis of purines without affecting bacterial adherence *in vitro* or in the bladder. However, the mutant strain was unable to increase in number into intracytoplasmic bacterial communities and not form biofilms in the YESCA medium (255).

In a recent article, the researchers used TraDIS-Xpress sequencing to analyse a mutant transposon library containing over 800,000 different mutants of *E. coli* strain BW25113. They found that the *purD*, *purH*, *purL* and *purE* genes, which are involved in the purine biosynthetic pathway, are also implicated in the fitness of the mature biofilm after 48 h of growth. *ΔpurD* was not able to form biofilms or micro-colonies. In addition, *ΔpurD* and *ΔpurE* showed deficiencies in biomass and curli fimbriae production, highlighting the importance of the purine biosynthetic pathway for matrix and curli fimbriae production in mature biofilms (256).

Similar results were found in studies with *S. aureus*. Li *et al.* found that a *ΔpurF* MRSA strain has a significantly lower biofilm formation ability and lower levels of eDNA than the isogenic strain. However, biofilm formation was restored by the addition of exogenous c-di-AMP (257). Likewise, Gélinas *et al.* found that the *de novo* purine biosynthetic pathway is upregulated in biofilms of *S. aureus* and *E. faecalis.* Furthermore, disruption of the *purA*, *purH*, and *purQ* genes in *S. aureus* significantly reduced biofilm production, but the addition of IMP restored the ability to form biofilms. They also found that the reduction in biofilm formation of the mutant strains was not due to a delayed or reduced cell division rate (258).

Additional proteomic studies in wt and Tn263 strains revealed differences in 13 proteins. The most significant differences were observed in

the proteins DnaK, GroEL, Pta, PtsI and outer membrane protein A (OmpA), most of which were previously associated with biofilm formation. Arita-Morioka *et al.* demonstrated that the *ΔdnaK* mutant strain could not form curli fimbriae and biofilms, as determined by the CR assay in YESCA medium at 30 °C (259), because DnaK may control the expression of RpoS and CsgD, proteins essential for curli-dependent biofilm formation (260). Moreover, the study by Wu *et al.* showed that the *ΔptsI* strain of avian pathogenic *E. coli* exhibited a different phenotype on CR agar, lower motility, and lower biofilm formation ability (261). Furthermore, OmpA is involved in biofilm formation, infection of eukaryotic cells, antibiotic resistance, and immunomodulation in Gram-negative bacteria (262).

Our transcriptomic analysis revealed three genes underexpressed (*dnaK, groL* and *adhE*), six genes overexpressed (*lptD, cysJ, pta, ilvC, elbB* and *ptsI*), and four genes with similar expression levels (*tsf, ftsZ, ompA* and *purL*) in the Tn263 mutant compared to the wild-type strain. Genes with different expression levels were also tested in biofilm-forming and non-biofilm-forming clinical strains. Although we found that the *adhE* and *ptsI* genes were overexpressed in clinical non-biofilm-forming strains, there was no concordance between the transcriptomic and proteomic results in mutant strain Tn263 and the comparison with non-biofilm forming strains. Thus, we conclude that the differences in gene expression observed in the Tn263 mutant may be exclusively related to the *purL* gene mutation and not to the non-biofilm phenotype *per se*.

In this regard, our results provide strong evidence that the *purL* gene is involved in curli-dependent biofilm formation. Thus, nucleotide biosynthetic pathway genes may be excellent candidates for potential antibiofilm compounds.

Having found a new potential target for the treatment of biofilms in *E. coli*, we wanted to analyse a fruit extract with antibacterial and antibiofilm activity, also in view of the worldwide concern about AMR.

AMR is a significant health problem, as available antibiotics are often ineffective against MDR bacteria. Even more worrisome is the lack of new antibiotics to combat these "superbugs" (203) and the rapid spread of ARGs, which could make bacterial pathogens much more lethal in the future than they are today (263).

According to the World Health Organisation (WHO) annual report on antibacterial agents in clinical and preclinical development, 43 new antibiotics and 27 non-traditional antibacterial agents have been identified in phase 1-3 trials and are currently under investigation. Of these 43 antibiotics, 26 are effective against the priority pathogens listed by the WHO. However, the recently approved antibiotics are insufficient to combat the increasing prevalence of AMR (264). The WHO also reports two antibiofilm agents in clinical trials. Among them, the engineered cationic peptide PLG0206 (WLBU2), with broad-spectrum activity and tested against MRSA and methicillin-susceptible *S. aureus* (MSSA) biofilms, is currently in phase 1 trial recruiting. In addition, the alginate oligosaccharide (G-block) fragment, extracted and purified from the marine algae *Laminaria hyperborean*, started phase 2 trials in 2018 as a treatment for infections related to cystic fibrosis (264).

Herbal remedies have been used by humanity for centuries, and some of them are essential for the prevention and treatment of infectious diseases. In this context, the Chinese herbs *Scutellara baicalensis*, *Glycyrrhiza glabra*, *Taraxacum officinale*, and *Tussilago farfara* showed antibacterial properties against *S. aureus*. On the other hand, *S. baicalensis* inhibited MSSA and MRSA biofilms, and *G. glabra* showed antimicrobial activity against *E. faecalis* (265).

As explained in the Introduction, section 3, other new antibiofilm agents of natural origin, including those with proteolytic activity such as our extract, are currently being investigated and have shown promising perspectives. However, many properties of the extracts are not yet well characterised, such as the molecular structure of the bioactive compounds (27), which delays the development of better antibiofilm agents. Importantly, due to their proteolytic activity, these extracts are designed to destroy

biofilms by degrading the EPS matrix and, in combination with conventional antibiotics, they can also have a synergistic effect by allowing the antibiotics to penetrate the biofilm (266,267).

Among the most studied proteases of natural origin, we found papain, a cysteine protease derived from papaya fruit (*C. papaya*). Baidamshina *et al.* studied this extract and found that soluble and immobilised papain has antibiofilm activity against biofilms formed by *S. aureus* and *S. epidermidis*. Papain has synergistic effects with ciprofloxacin and gentamicin against *S. epidermidis* biofilms (268). Papain has been used for the safe degradation of necrotic tissues because it does not affect healthy tissue due to its inactivation by α1-antitrypsin. Papain and fruit-derived proteases are, thus, important agents for wound healing, whether or not they are affected by bacterial biofilms (268).

The anti-plaque and anti-gingivitis effects of papain have also been studied, showing more significant results when used as a toothpaste combined with bromelain, miswak, and neem (269). In addition, the study by Mugita *et al.* found that actinidin, a cysteine protease from kiwi, significantly removed the tongue coating in elderly subjects, and trypsin, papain, and actinidin reduced *in vitro* monospecies and multispecies biofilms (113). Kiwi fruit promotes gut health and has anti-oxidative, anti-proliferative, anti-inflammatory, antimicrobial, antihypertensive, anti-hypercholesterolemic, neuroprotective and anti-obese properties (270). Moreover, green and red kiwi (pulp and peel) have shown antimicrobial activity against *P. aeruginosa*, *E. coli*, *Listeria monocytogenes* and *S. aureus* (271).

In **manuscript 1**, we summarised the results obtained when we tested a fruit extract with proteolytic activity against different species of bacteria, including Gram-positive and Gram-negative strains. All the strains were biofilm-formers and were involved in different infections.

We found that this fruit extract showed an antibacterial effect and disrupted mature biofilms. In addition, we demonstrated its bactericidal effect at 2x minimum inhibitory concentration (MIC) and 4x MIC after 24 h

of incubation. However, the IC_{50} value obtained with the XTT assay in Jurkat cells to determine the cytotoxicity of the extract is inaccurate due to the viscosity and turbidity of the fruit extract.

Although the MIC and MBEC values obtained are encouraging, they could be improved if we could test the active molecule of the fruit extract in our strains. Once this is achieved, the fruit extract with proteolytic enzymes could be a promising new antimicrobial and antibiofilm agent. In comparison to our results, Almeida *et al.* found that *Actinidia arguta* (hardy kiwifruit) hydroalcoholic and alcoholic extracts showed antimicrobial activity against *S. aureus* with MIC values of 50 mg/mL (272). In a few cases, this MIC value was higher than the MIC value we obtained, indicating the promising future for the fruit extract we analysed.

Our extract also showed great activity against some of the species involved in periodontal disease. For example, some studies have shown that *E. faecalis* is the most commonly detected species in root canal–treated teeth (241,273). In addition, *S. mutans* is one of the most common cariogenic pathogens (274), and *E. coli, K. pneumoniae* and other *Enterobacterales* are associated with periodontitis (275). Therefore, the active molecule of our fruit extract could be tested for use in toothpaste or mouthwash to prevent and combat plaque and other oral diseases.

To our knowledge, there is no evidence of the *in vivo* activity of proteases against biofilm-related diseases of the gallbladder and gut caused by *Salmonella* sp. and *Shigella* sp. However, several compounds have shown antimicrobial activity against these pathogens. Chloroform extract of *Lawsonia inermis* showed antimicrobial properties against *Shigella* and *Salmonella* strains (276). Additionally, an extract of olive oil polyphenols showed antimicrobial activity against *S. enterica* serovar Typhimurium and *S. aureus* (277). Furthermore, 10 plant polyphenols (epigallocatechin, epigallocatechin-3-O-gallate, punicalagin, tannic acid, castalagin, prodelphinidin, geraniin, procyanidins, a mixture of black tea, and green tea polyphenols) were tested against some foodborne pathogens and found to have antimicrobial activity against *S. aureus*, *Salmonella*, *E. coli,* and *Vibrio*

(278). Similarly, Puupponen-Pimiä *et al.* found that cloudberry, raspberry, and strawberry extracts were potent inhibitors against *Salmonella* strains (279).

It is, therefore, worth noting that while hundreds of innovative and promising molecules are being researched to combat AMR and biofilms, their final development requires financial support, which is difficult to obtain, delaying the progress of better antimicrobial and antibiofilm agents. Meanwhile, we also wanted to explore the association between virulence and antimicrobial resistance genes with biofilm formation, which could also be an effective strategy for finding other targets to combat biofilms.

The study of biofilms formed mainly by bacteria of the ESKAPE group and the list of priority pathogens from the WHO is, thus, attracting increasing interest. *K. pneumoniae* and *E. coli* are two Gram-negative bacteria that belong to the list, and are involved in many infections. Characterising strains of these species in terms of virulence and resistance, and studying the relationship of these factors whit biofilm formation, may facilitate the search for new strategies to inhibit or eradicate biofilms.

In paper 3, we determined the carriage of a series of VFGs and ARGs among 127 clinical *K. pneumoniae* strains isolated from urine, the respiratory tract, and blood samples. VFGs included genes encoding adhesins, iron recruitment systems, and toxins. ARGs included ESBL-associated genes (bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, bla_{OXA48}), genes related to resistance to quinolones [*aac (6)-Ib-cr*, *qnrB*], aminoglycosides (*aadb*), and sulfonamides (*sul1* and *sul2*). Serum resistance, string test, and biofilm formation were also determined.

Our results showed great similarities with the study by El Fertas-Aissani *et al.* (280). They found similar percentages of several VFGs, including genes encoding fimbrial adhesins type 1 and type 3 (*fimH-1* and *mrkD)* and genes encoding the siderophores enterobactin (*entB)*, ferric-catecholate receptor (*iroN)*, aerobactin (*iucA*), and yersiniabactin (*ybtS* and *fyuA*). These comparisons showed that fimbriae and enterobactin are present in virtually all clinical strains of *K. pneumoniae*. Furthermore, an increasing rate of

yersiniabactin was observed by El Fertas-Aissani *et al.* They compared these values (46.3%) with previous studies in which yersiniabactin was only detected in almost 20% of isolates. Nevertheless, some differences were also observed in comparison with our study. El Fertas-Aissani *et al.* significantly associated yersiniabactin with strains from the bloodstream (81.8%) and lungs (75%); however, we found less than 20% of this siderophore in each group of isolates (blood, the respiratory tract, and urine). We also found the *rmpA* gene (7.1%) and the HMV-phenotype (13.4%) in a significant proportion in our study.

As observed in previous studies, a considerable prevalence of yersiniabactin-encoding genes among respiratory tract isolates was found, confirming the crucial role of yersiniabactin in pulmonary infections (281). As in the study by Tanni *et al.* (282), we found a statistically significant association between the capsule-associated *uge* gene (UDP galacturonate 4-epimerase) and the origin of the isolates, with the majority of urine isolates harbouring this gene. Several studies have shown that mutant strains of *K. pneumoniae* in which the *uge* gene was deleted, were less virulent and less able to cause UTI, pneumonia, or sepsis (145,149,150).

It is important to highlight the global increase in hvKp strains and their impact on health. Although several studies define hvKp as a string test-positive strain, not all hvKp strains are HMV, and thus, there is no unified categorisation (283). Some biomarkers have been proposed to classify hvKp, of which the *rmpA* and *iucA* genes are the most significant (284). Aerobactin is one of the major siderophores in hvKP. Russo *et al.* demonstrated that aerobactin-deficient hvKp mutant strains exhibited reduced virulence in mice, confirming the key role of aerobactin in hvKp virulence (285). In the present work, we classified 24 strains (18.9%) as hvKp. Of these, 13 strains were susceptible to all antimicrobials tested, nine were ESBL producers, one was resistant to fosfomycin and one was MDR. The first reports investigating hvKp resistance found low resistance to antimicrobials and less than 5% of ESBL producers (286). Currently, antimicrobial-resistant hvKp strains have increasingly emerged, as observed in the study conducted by Li *et al.*, in

which 24.5% of the strains examined were classified as hvKp and 57% were KPC-producing isolates, emphasising the importance of epidemiological surveillance and clinical knowledge of this pathogen (287). Similarly, reports from China, the United States and Argentina on carbapenemase-producing hvKp strains have indicated an increase in hypervirulence and multidrug resistance, leading to an increase in mortality by this pathogen (288–291).

Another growing concern in health research is the alarming increase of colibactin-positive Gram-negative bacteria. Although we found only two colibactin-positive strains in our study, this toxin is being investigated in several species for its association with DNA damage and host cell cycle disruption. Deletion of *clbA* in the *K. pneumoniae* strain K1 CC23 abolished colibactin production and prevented the development of meningitis in a mouse model (292). In addition, colibactin production has been linked to yersiniabactin production at the biosynthetic level, being part of an ICE in *K. pneumoniae* that allows horizontal transference of both virulence determinants (293).

Regarding biofilm formation, several studies found a similar or even higher percentage of biofilm-forming strains than those we observed (80.3%), suggesting that clinical *K. pneumoniae* strains have a high capacity for biofilm formation (280,294–296). However, in contrast to El Fertas-Aissani *et al.* (280), we found a correlation between biofilm formation and the origin of the isolates, being the urine strains more biofilm-formers than the isolates from the bloodstream or the respiratory tract.

We also observed that HMV strains had a lower ability to form biofilms. This fact can be explained by the ability of the hyper-capsule to hide type 3 and type 1 fimbriae, thus preventing adhesion. This was also hypothesised in the study published by Cubero *et al.* in which HMV K1 isolates showed low initial adhesion despite the expression of the *mrkD* and *fimH* genes (297).

Concerning AMR, we found that 40% of strains had a MDR phenotype, and almost half were isolated from urine. Although this seems to

be a noteworthy percentage, other studies found even higher percentages of MDR (296,298) and carbapenemase-producing *K. pneumoniae* strains (296), which could be due to the different geographic areas in which each study was conducted. However, compared to the European Antimicrobial Resistance Surveillance Network (EARS-Net) report, we have a more significant percentage of MDR strains. The report indicates that, in 2018, more than one-third (37.2%) of *K. pneumoniae* isolates were resistant to at least one of the antimicrobial groups that are regularly monitored (i.e., fluoroquinolones, third-generation cephalosporins, aminoglycosides and carbapenems). In addition, they mentioned that there were significant trends of increasing resistance rates to fluoroquinolone and carbapenems. In the case of Spain, the report states an alarming increase in the percentage of resistance to third-generation cephalosporins, carbapenems and aminoglycosides between 2015 and 2018, coinciding with the finding of this study (299).

Among the ESBL production, we found that 43.3% of the strains were ESBL producers, and 10.2% were carbapenemase producers. Furthermore, urine isolates were more likely to be ESBL-producing than respiratory or blood strains.

After studying *K. pneumoniae* strains, in **paper 4** we characterised 376 ExPEC strains to investigate the relationship between the ability to form biofilms and antimicrobial resistance or virulence factors.

According to recently published statistical models, an estimated 4.95 million deaths were associated with AMR in 2019, including 1.27 million deaths attributable to AMR (251). Antibiotic misuse will continue to increase these rates each year as new antimicrobial therapies become harder to find. The antimicrobial resistance profiles in our collection showed the highest percentages of resistance to ciprofloxacin (48.7%), trimethoprim-sulfamethoxazole (47.9%), and ampicillin (38%). The EARS-Net reports that more than half (58.3%) of *E. coli* isolated in the European Union and European Economic Area in 2018 were resistant to at least one of the regularly monitored antimicrobial groups (299). In Spain, there was a significant increase in resistance to third-generation cephalosporins, and resistance to

carbapenems remained rare, as was also observed in our study. In addition, our results showed a lower percentage of ampicillin resistance, but a higher percentage of ciprofloxacin and third-generation cephalosporins resistance compared to the EARS-Net report. Consequently, we found a significant percentage of strains with ESBL-encoding genes, being *bla*$_{CTX-M-15}$ the most common.

In our study, 38% of the isolates were classified as MDR. Nevertheless, a direct correlation between antimicrobial susceptibility and the ability to form biofilms was found, although it was not statistically significant. Interestingly, non-biofilm-forming strains had a higher percentage of resistance to ciprofloxacin than biofilm-forming strains. Previous studies have shown that quinolone resistance was associated with a decrease in biofilm formation in *Salmonella enterica* strains (300) and that quinolone-resistant *E. coli* strains were less virulent than quinolone-susceptible strains (301).

Regarding classification by phylogenetic groups, isolates belonging to phylogenetic groups B2 and D had a stronger ability to form biofilms than the other phylogenetic groups, which is consistent with other similar published studies (302–304). However, in contrast to the previously studied *K. pneumoniae* strains, no statistical significance between the origin of the strains and biofilm formation was found, which is consistent with the study by El-Baky *et al.* (305).

Compared to the study by Katongole *et al.*, we found a higher percentage of biofilm-forming strains. Surprisingly, they found no correlation between biofilm formation and the virulence factors or antimicrobials tested (306). In contrast to them, a statistically significant correlation between biofilm formation and the presence of various siderophores, adhesins or toxins was observed in our study. The *iroN* gene encoding the salmochelin receptor, the gene encoding type 1 fimbriae (*fimH*), the *sfa* and *papA* genes encoding adhesins, the *hlyA* and *cnf1* genes encoding toxins, and the genes encoding colibactin were statistically correlated with biofilm formation. In accordance with our findings, Magistro *et al.*, observed

that the *iroN* gene was strongly upregulated during biofilm formation by ExPEC, and its disruption reduced the ability to form biofilms by 50% (307). Furthermore, the IroN receptor was shown to contribute to the invasion of urothelial cells by ExPEC strains (308). As expected, the *fimH* gene was the major determinant of biofilm formation and was found in 92% of the strains analysed. Additionally, the adhesin genes *sfa* and *papA*, encoding S-fimbriae and P-fimbria, respectively, were mainly associated with UPEC. Zamani *et al.* also found a higher prevalence of these genes in moderate or strong biofilm-producing strains than in weak or non-biofilm producers (309).

Among the toxins, we found that α-haemolysin and cytotoxic necrotising factor 1 were associated with biofilm formation. However, one of the most remarkable findings was the statistically significant relationship between colibactin-encoding genes and biofilm formation. In the study conducted by Suresh *et al.*, they found that 7.6% of the strains harboured the *pks*-genomic island, and the majority of these strains had high biofilm formation ability and a low AMR rate (310). In our study, a higher percentage of colibactin-positive *E. coli* strains was found. Most were biofilm-formers, belonged to phylogenetic group B2, and had lower AMR rates, higher clonal diversity, and high relatedness to most of the VFGs studied. A previous analysis of the *pks*-genomic island revealed that it is associated with HPI, which carries the yersiniabactin gene cluster, suggesting an interaction between the two pathways (311). In addition, Martin *et al.* investigated the role of the PPTase ClbA through functional interchangeability between EntD and ClbA. They found that ClbA contributes to the siderophore biosynthetic pathways and that its presence is required to maintain the virulence of ExPEC in a mouse sepsis model (312).

As discussed in the Introduction, section 4.1.5.3, colibactin-positive *E. coli* strains can cause DNA damage and genomic instability in mammalian cells (157). This toxin has been linked to colorectal cancer in humans (158), and some studies have also shown its crucial role in the pathogenesis of UTIs by inducing DNA damage in urothelial cells (313). In addition, the role of the *clbG* in colibactin synthesis and the initiation of meningitis was demonstrated

in the study by Wang *et al.* in which deletion of *clbG* in the avian pathogenic *E. coli* XM did not result in *in vitro* megalocytosis or cell cycle arrest. Therefore, none of the mice infected with the mutant strain developed meningitis or any of the associated symptoms (314).

Interestingly, the probiotic *E. coli* Nissle 1917 harbours colibactin, but this strain has been shown to require colibactin for its anti-inflammatory properties. Olier *et al.* inactivated the *clbA* gene in this strain to obtain a colibactin mutant without genotoxic activity. However, they found that inactivation of the colibactin biosynthetic pathway also eliminated the probiotic activity of the strain (315).

Mesalamine (5-aminosalicylic acid) has been suggested to target colibactin and some of its associated diseases (inflammatory bowel disease and colorectal cancer). Mesalamine showed an inhibitory effect on the production of intestinal inflammatory mediators, the proliferation of colorectal cancer cells and limiting the amount of *E. coli* in the gut microbiota, thereby reducing colibactin production and its associated properties, including biofilm formation and antibiotic resistance (316). On the other hand, the effect of the cinnamon extract was studied by Kosari *et al.* who found that sub-MIC concentrations of cinnamaldehyde and cinnamon essential oil affected *clbB* gene expression compared to untreated strains, altering also the biofilm formation in *E. coli* strains isolated from patients diagnosed with colorectal cancer (317).

The role of colibactin in biofilms and its importance in several infectious diseases need further research. Biofilms formed by *E. coli* and other priority pathogens also require additional knowledge, as yet unidentified environmental or other genetic factors may also be involved in this process. Additionally, epidemiological surveillance is needed to detect antibiotic resistance and virulence factors to prevent the spread of strains with hypervirulent pathotypes that pose a real threat to susceptible populations, especially when these pathotypes carry ARGs.

X. Conclusions

1. Competitive index (CI) and viable cell quantification indicate competitive interaction between *E. faecalis* and *K. pneumoniae* in polymicrobial biofilms when co-cultured in a glucose-enriched medium.

2. Although *K. pneumoniae* has a stronger adhesion capacity than *E. faecalis*, this property was not decisive for the further development of the polymicrobial biofilm formed by both species.

3. The inhibitory effect of *E. faecalis* on *K. pneumoniae* was not due to the production of bacteriocins but to the lactic acid produced by *E. faecalis*, which inhibited the growth of *K. pneumoniae* under conditions of excess glucose and limited oxygen.

4. The tested fruit extract showed antibacterial activity and was able to disrupt mature biofilms formed by various Gram-positive and Gram-negative bacterial species. If the active molecule can be isolated from this extract, it could be a good candidate as an antibiofilm agent.

5. The *purL* gene is essential for biofilm formation mediated by curli fimbriae in *E. coli* and could be considered a potential antibiofilm target.

6. The Tn263 and *ΔpurL::cat* mutant strains lost the ability to form biofilms mediated by curli fimbriae on M63 broth. However, they regained this ability when the medium was enriched with inosine.

7. An insertion in the N-terminal domain of the *purL* gene affected the purine biosynthetic pathway and the expression of 13 proteins, including AdhE, CysJ, DnaK, ElbB, FtsZ, GroL, IlvC, LpD, MgtA, OmpA, Pta, PtsI and Tsf.

8. In the collection of strains under study, urinary *K. pneumoniae* strains were more resistant to antimicrobials, produced more ESBL, and more biofilm-formers "*in vitro*" than the respiratory or blood strains.

9. The *uge* gene was significantly associated with urinary strains and yersiniabactin was significantly associated with strains isolated from the respiratory tract, which indicates the relationship of these genes to the niche in which the bacteria carrying the gene are found.

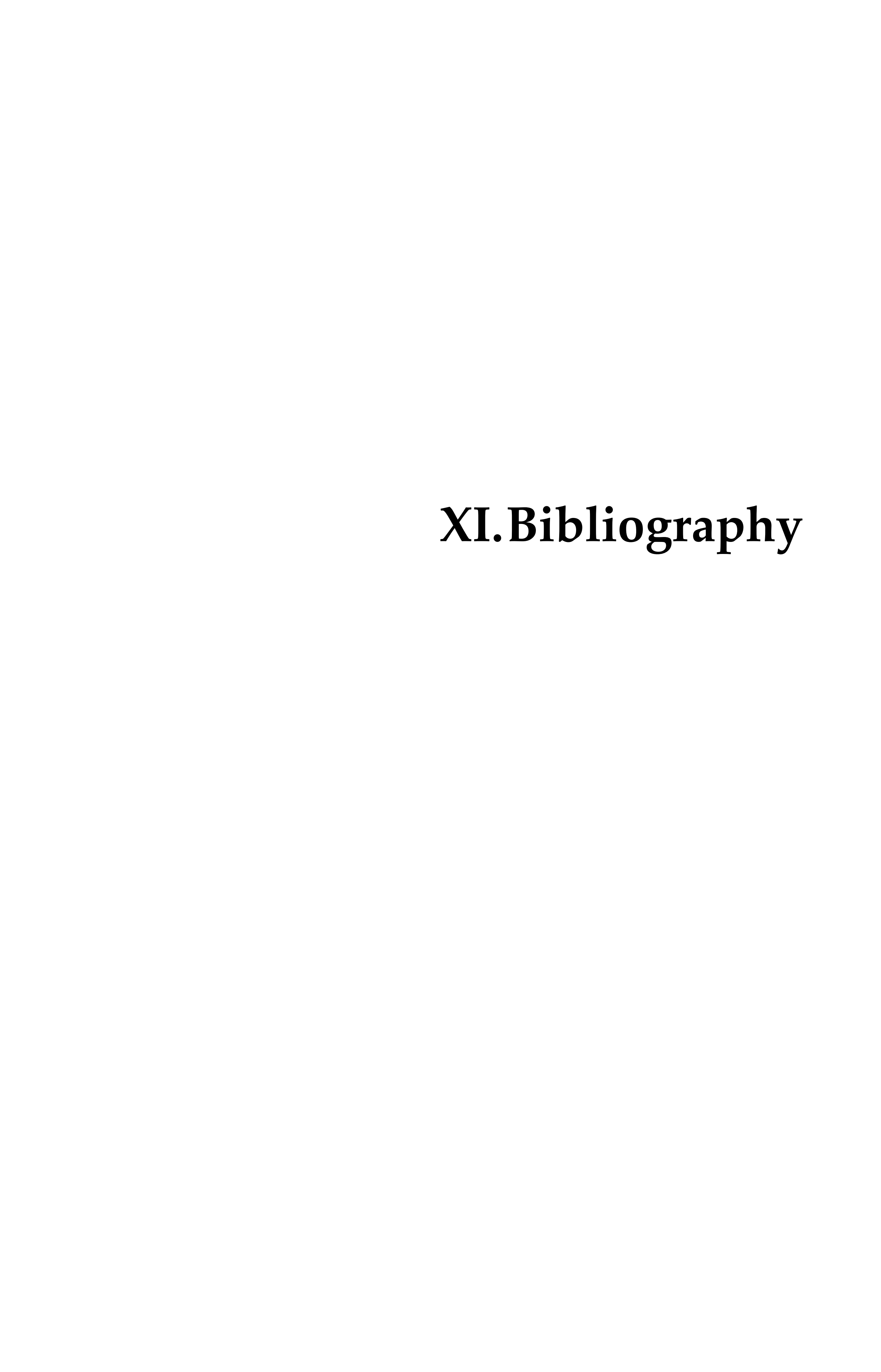
10. An inverse relationship between antimicrobial resistance and virulence was observed with the *K. pneumoniae* and *E. coli* strains analysed. Thus, the most resistant strains showed a low prevalence of virulence genes and vice versa.

11. The acquisition of ciprofloxacin-resistance seems to affect the ability of *E. coli* strains to form biofilms, which confirms previous studies carried out in our laboratory.

12. The presence of colibactin-encoding genes was statistically correlated with biofilm formation in *E. coli* strains. Since this toxin is linked to the development of colorectal cancer, further study of the relationship between the presence of this toxin, biofilm formation and this type of cancer should be performed.

13. Biofilms formed by *E. coli*, *K. pneumoniae* and other priority pathogens require additional knowledge, as yet unidentified genetic factors may be involved in the process of biofilm formation.

14. Epidemiological surveillance should be mandatory worldwide to detect circulating clones that pose a threat to susceptible populations, especially when these pathotypes carry ARGs and VFGs of high risk.



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