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Genomic and proteomic study of antimicrobial resistance in *Escherichia coli* and *Enterococcus* spp. recovered from wild animals

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University of Trás-os-Montes and Alto Douro
School of Life and Environmental Sciences

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University of Trás-os-Montes and Alto Douro,

___/___/_____

Hajer Radhouani

TO MY PARENTS

إِلىَ وَالِدِي سَلْوَى وَ عَبْدَالْحَمِيدْ

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إلى والداي، أخى وأختى لا يمكنني حقيقة أن أعبر عن خالص تقديري لكم على كل الدعم والحب الذي قدمتموه لي. وما أمكنني الوصول إلى ما واصلت إليه دون دعمكم. وقد كنتم دائما السند الدعم تجدون الكلمات المناسبة التي تجعلني أتجاوز الصعاب. آمنتم بي أدين لكم بنجاح بكل شيء.

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ABSTRACT

Genomics, proteomics and bioinformatics tools allow for more in-depth knowledge about the physiology and structure of bacteria and also about mechanisms involved in antimicrobial resistance. Antimicrobial resistance, evolving and spreading among bacteria, poses a serious and growing threat to public health. Though, resistance has also been detected in the absence of antimicrobial exposure, such as in bacteria from wildlife, raising a question about the mechanisms of occurrence and persistence of resistant strains under similar conditions, and the implications for resistance control strategies.

In order to study antimicrobial resistance in *Escherichia coli* and *Enterococcus* spp., 151 faecal samples were collected from three wild animal species, seagulls (*Larus cachinnans*, 57), common buzzards (*Buteo buteo*, 42) and red foxes (*Vulpes vulpes*, 52). Seagull samples were recovered from the Berlengas nature reserve, those of common buzzards from the Peneda Gerês natural park and other rural conservation areas of Portugal and those of red foxes were collected in the north of Portugal during red fox hunts. A total of 111 *E. coli* (73.5%) and 135 enterococci (89.4%) were isolated from Levine and Slanetz-Bartley plates without antimicrobial agents. After isolation in selective plates supplemented with cefotaxime (CTX) and vancomycin, respectively, the occurrence of extended-spectrum-β-lactamase (ESBL)-producing *E. coli* was 12.7% (seagulls, 19.3%; common buzzards, 15.2%; red foxes, 4%) and the prevalence of vancomycin-resistant enterococci (VRE) was 20.4% (seagulls, 10.5%; common buzzards, 36.4%; red foxes, 21.1%).

All CTX-resistant *E. coli* isolates exhibited a resistance phenotype to cefotaxime and/or ceftazidime and had a positive screening test for ESBL production. The most predominant β-lactamase genes were the $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-32}}$ genes (34.8%) together with the $bla_{\text{CTX-M-1}}$ (17.4%), $bla_{\text{SHV-12}}$ (8.7%), $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-14a}}$ (4.3%) genes. Most of the ESBL and non-ESBL *E. coli* isolates were multiresistant, with high levels of resistance to tetracycline (57.5%), streptomycin (53%) and sulfamethoxazole/trimethoprim (35.1%), due to the presence of *tet*(A) and/or *tet*(B) genes (81.7%), *aad*A gene (45.1%) and different combinations of *sul* genes (97.8%), respectively. The most common virulence factor gene was *fim*A (63.3%) and the majority of the *E. coli* isolates belonged to phylogenetic groups A (39.1%) and B1 (27.3%). Regarding to the VRE and non-VRE isolates, *E. faecium* and *E. faecalis* were the most predominant enterococcal species. High levels of tetracycline and erythromycin resistance were detected and resistance gene profiles were diverse amongst these isolates; the most prevalent genotype was *tet*(M)+*tet*(L)+*erm*(B). Concerning virulence factors, the *gelE*, *hyl*, *cpd*, *esp* and *agg* genes were the most predominant. VRE isolates with acquired vancomycin resistance (*van*A genotype) were found in 11.3% of the faecal samples analysed; and 9.1% contained VRE isolates with intrinsic vancomycin resistance (*van*C1 genotype). The *van*A-*E. faecium* isolates belonged to multilocus sequence types ST273 and ST262 (clonal complex CC17) and also to ST5.

ESBL-*E. coli* and *van*A-containing enterococci were also studied through a detailed proteomic approach consisting in two-dimensional gel electrophoresis (2-DE) followed by matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI/TOF-MS). A total of 686 spots were excised from 2-DE gels of 3 ESBL-*E. coli* and 4 VRE strains from which 562 proteins were successfully identified (81.9%) and fully characterized through database interrogations. The proteins identified were associated to different biological functions such as glycolysis, transport, biosynthesis, antimicrobial resistance, among others. The results also indicated in *E. coli* and enterococci strains the presence of proteins involved in stress response, as chaperone proteins DnaK, WrbA, GroEL, among others. Additionally, the VanA protein (Mw: 37419,10938 /

PI: 5,79), which prevents vancomycin/teicoplanin binding, was identified when studying the whole-cell proteomic profile of *van*A-containing enterococci strains. This approach showed that it is possible to evaluate protein profiles in resistant bacteria and in response to antimicrobial stress conditions to further understand the associated resistance mechanisms. Quantitative proteomic analyses on specific protein classes or subproteomes in ESBL-*E. coli* and VRE strains will provide new functional insights into antimicrobial resistance mechanisms facilitating the identification of diagnostic or prognostic disease markers and the discovery of therapeutic targets.

This thesis discussed the prevalence and spread of antimicrobial resistance in indicator bacteria such as *E. coli* and enterococci in wild animals and showed that wildlife may be an important reservoir of antimicrobial-resistant bacteria and resistance genes. Moreover, this research showed the presence of strains carrying virulence factor genes and belonged to high-risk clonal complexes, which adds to the public health concerns that arise from the spread of ESBL-*E. coli* and VRE into wildlife.

KEYWORDS: Antimicrobial resistance, extended-spectrum β-lactamases-producing *Escherichia coli*, genomics, proteomics, vancomycin-resistant enterococci, wild animals.

| RESUMO

As ferramentas genómicas, proteómicas e bioinformáticas permitem um conhecimento mais aprofundado sobre a fisiologia e estrutura bacterianas e também dos mecanismos envolvidos na resistência antimicrobiana. A resistência aos antimicrobianos tem evoluído e disseminado entre bactérias, representando uma séria e crescente ameaça para a saúde pública. No entanto, esta resistência tem sido detectada também na ausência de exposição antimicrobiana em animais selvagens, suscitando questões acerca dos mecanismos de ocorrência e persistência de estirpes resistentes sob condições similares, e as implicações para estratégias de controlo de resistência.

Com o objectivo de estudar a resistência antimicrobiana em *Escherichia coli* e *Enterococcus* spp., foram recolhidas 151 amostras fecais de três espécies de animais selvagens nomeadamente gaivotas-de-patas-amarelas (*Larus cachinnans*, 57), águias-de-asa-redonda (*Buteo buteo*, 42) e raposas-vermelhas (*Vulpes vulpes*, 52). As amostras de gaivota foram recolhidas na reserva natural das Berlengas, as de águia no parque nacional da Peneda-Gerês e em outras áreas de conservação de Portugal e as amostras de raposa foram recolhidas no Norte de Portugal durante o período sazonal de caça. Através de placas de Levine e Slanetz-Bartley sem antibiótico foram isolados um total de 111 *E. coli* (73,5%) e 135 enterococos (89,4%). Após o isolamento em placas selectivas suplementadas com cefotaxima (CTX) e vancomicina, respectivamente, a ocorrência de *E. coli* produtoras de β-lactamases de amplo espectro (BLAE) foi de 12,7% (gaivotas, 19,3%; águias, 15,2%; raposas, 4%) e a prevalência de enterococos resistentes à vancomicina (ERV) foi de 20,4% (gaivotas, 10,5%; águias, 36,4%; raposas, 21,1%).

Todos os isolados de *E. coli* CTX-resistentes revelaram um fenótipo de resistência à cefotaxima e/ou à ceftazidima, apresentando um resultado positivo para a produção de BLAE. O genes da β-lactamase mais predominante foram os genes $bla_{\text{TEM-52}}$ e $bla_{\text{CTXM-32}}$ (34,8%) juntamente com os genes $bla_{\text{CTX-M-1}}$ (17,4%), $bla_{\text{SHV-12}}$ (8,7%), $bla_{\text{OXA-1}}$ e $bla_{\text{CTX-M-14a}}$ (4,3%). A maioria dos isolados de *E. coli* produtores e não produtores de BLAE foram multirresistentes, demonstrando níveis elevados de resistência à tetraciclina (57,5%), estreptomicina (53%) e sulfametoxazol/trimetoprim (35,1%), devido à presença dos genes *tet*(A) e/ou *tet*(B) (81,7%), o gene *aad*A (45,1%) e a diferentes combinações de genes *sul* (97,8%), respectivamente. O gene de virulência mais comum foi o *fim*A (63,3%) sendo que a maioria dos isolados de *E. coli* pertenciam aos grupos filogenéticos A (39,1%) e B1 (27,3%). Entre os isolados de enterococos resistentes e não resistentes à vancomicina, as espécies mais comuns foram *E. faecium* e *E. faecalis*. Foram detectados níveis elevados de resistência à tetraciclina e à eritromicina e os perfis genotípicos de resistência foram diversos nestes isolados, o genótipo mais comum foi *tet*(M)+*tet*(L)+*erm*(B). No que respeita a factores de virulência sendo os genes *gelE*, *hyl*, *cpd*, *esp* e *agg* foram os mais predominantes. Foram encontrados isolados de ERV com resistência adquirida à vancomicina (genótipo *van*A) em 11,3% das amostras fecais analisadas; e 9,1% das amostras apresentaram ERV com resistência intrínseca à vancomicina (genótipo *van*C1). Através da análise por MLST (Multilocus Sequence Typing) conclui-se que os isolados *van*A-*E. faecium* pertencam ao ST273 e ST262 (complexo clonal CC17) e também ao ST5.

As estirpes de *E. coli* produtoras de BLAE e de enterococos com *van*A foram também estudadas através de uma abordagem proteómica detalhada abrangendo electroforese bi-dimensional (E2D) e identificação por espectrometria de massa com ionização a laser assistida por matriz com analisador tempo de voo (MALDI-TOF MS). Um total de 686 *spots* foram excisados a partir dos geis E2D de 4 estirpes de *E. coli* produtores de BLAE e 3 estirpes de ERV dos quais 562 proteínas foram identificadas com sucesso (81,9%) e caracterizadas por interrogação de base dados. As

proteínas identificadas foram associadas a diferentes funções biológicas, tais como glicólise, transporte, biossíntese, resistência aos antibióticos, entre outros. Os resultados obtidos em *E. coli* e enterococos revelaram a presença de proteínas envolvidas na resposta ao stress, tais como as proteínas *chaperone* DnaK, WrbA, GroL, entre outras. Adicionalmente, a proteína VanA (MM: 37419,10938 / PI: 5,79), que evita a ligação da vancomicina/teicoplanina, foi identificada ao analisar o proteoma total de estirpes de enterococos *van*A. A descrição detalhada das proteínas identificadas em estirpes BLAE e ERV pode proporcionar novos alvos para o desenvolvimento de agentes antimicrobianos. Esta abordagem revelou perfis proteicos de bactérias resistentes e em resposta a várias condições de stress antimicrobiano que possibilitaram um maior conhecimento dos mecanismos de resistência associados. A análise proteómica quantitativa em classes específicas de proteínas ou subproteomas em estirpes de ERV e de *E. coli* produtoras de BLAE abrirá novas perspectivas funcionais no estudo de mecanismos de resistência antimicrobiana facilitando a identificação de marcadores de diagnóstico ou prognóstico e a descoberta de alvos terapêuticos.

Conclui-se que a prevalência e disseminação de bactérias indicadoras resistentes aos antimicrobianos, como *E. coli* e *Enterococcus* spp., em animais selvagens revelaram que estes podem representar um importante reservatório de bactérias resistentes e de genes de resistência. Este estudo revelou também a presença de estirpes portadoras de genes de virulência, pertencendo a complexos clonais de elevado risco, o que acresce às preocupações de saúde pública que surgem da disseminação de ERV e de *E. coli* produtoras de BLAE em ecossistemas selvagens.

PALAVRAS-CHAVE: Animais selvagens, enterococos resistentes à vancomicina, *Escherichia coli* produtoras de β-lactamases de amplo espectro, genómica, proteómica, resistência antimicrobiana.

| RÉSUMÉ

Les outils génomiques, protéomiques et bioinformatiques permettent une meilleure compréhension de la physiologie et de la structure bactérienne ainsi que des mécanismes impliqués dans la résistance aux antimicrobiens. L'évolution et la propagation de la résistance aux antimicrobiens chez les bactéries constituent une menace grave et croissante pour la santé publique. Toutefois, cette résistance a également été détectée en l'absence d'exposition aux antimicrobiens chez les bactéries provenant d'animaux sauvages, ce qui soulève la question des mécanismes d'apparition et de persistance des souches bactériennes résistantes dans des conditions similaires, et leurs implications dans les stratégies visant le contrôle de la résistance aux antimicrobiens.

Afin d'étudier la résistance aux antimicrobiens d'*Escherichia coli* et d'*Enterococcus* spp., 151 échantillons fécaux provenant de trois espèces d'animaux sauvages, les goélands leucophées (*Larus cachinnans*, 57), les buses variables (*Buteo buteo*, 42) et les renards roux (*Vulpes vulpes*, 52) ont été prélevés. Les échantillons de goélands ont été prélevés dans la réserve naturelle des Berlengas, ceux des buses dans le parc national du Peneda-Gerês ainsi que d'autres aires de conservation du Portugal et ceux des renards dans le nord du Portugal au cours de la saison de chasse. A travers des milieux de Levine et de Slanetz-Bartley sans antibiotiques, un total de 111 isolats d'*E. coli* (73,5%) et 135 d'entérocoques (89,4%) ont été isolés. Après isolement à travers des milieux sélectifs contenant de la céfotaxime (CTX) et la vancomycine, l'apparition respective des souches d'*E. coli* productrices de β-lactamases à spectre élargi (BLSE) a été de 12,7% (goélands, 19,3%; buses, 15,2%; renards, 4%) et la prévalence des isolats d'entérocoques résistants à la vancomycine (ERV) a été de 20,4% (goélands, 10,5%; buses, 36,4%; renards, 21,1%).

Tous les isolats d'*E. coli* résistants à la CTX ont montré un phénotype de résistance à la céfotaxime et/ou à la ceftazidime, indiquant un résultat positif à la production de BLSE. Les gènes de β-lactamase prédominants ont été ceux de $bla_{\text{CTX-M-52}}$ et $bla_{\text{TEM-32}}$ (34,8%) suivis des gènes $bla_{\text{CTX-M-1}}$ (17 4%), $bla_{\text{SHV-12}}$ (8,7%), $bla_{\text{OXA-1}}$ et $bla_{\text{CTX-M-14a}}$ (4,3%). La plupart des isolats d'*E. coli* producteurs et non-producteurs de BLSE étaient multirésistants, montrant des taux élevés de résistance à la tétracycline (57,5%), la streptomycine (53%) et le sulfaméthoxazole/triméthoprime (35,1%) en raison de la présence respectivement des gènes *tet*(A) et/ou *tet*(B) (81,7%), *aad*A (45,1%) et différentes combinaisons des gènes *sul* (97,8%). Le gène de virulence le plus common a été celui du *fim*A (63,3%) et la majorité des isolats d' *E. coli* appartenaient aux groupes phylogénétiques A (39,1%) et B1 (27,3%). Parmi les isolats d'entérocoques résistants et non résistants à la vancomycine, les espèces les plus communes ont été celles d'*E. faecium* et d'*E. faecalis*. Les isolats d'entérocoques ont présenté des niveaux élevés de résistance à la tétracycline et l'érythromycine et les profils génotypiques de résistance ont été divers chez ces isolats mais le plus fréquent a été celui de *tet*(M)+*tet*(L)+*erm*(B). En ce qui concerne les gènes de facteur de virulence, les gènes *gel*E, *hyl*, *cpd*, *esp* et agg ont été les plus répandus. Les isolats d'ERV ayant une résistance acquise à la vancomycine (génotype *van*A) ont été trouvés dans 11,3% des échantillons fécaux analysés, et 9,1% de ces échantillons ont présenté des isolats d'ERV avec une résistance intrinsèque à la vancomycine (génotype *van*C1). Les isolats *E. faecium van*A appartenaient aux séquences types ST273 et ST262 (du complexe clonal CC17) ainsi qu'au ST5.

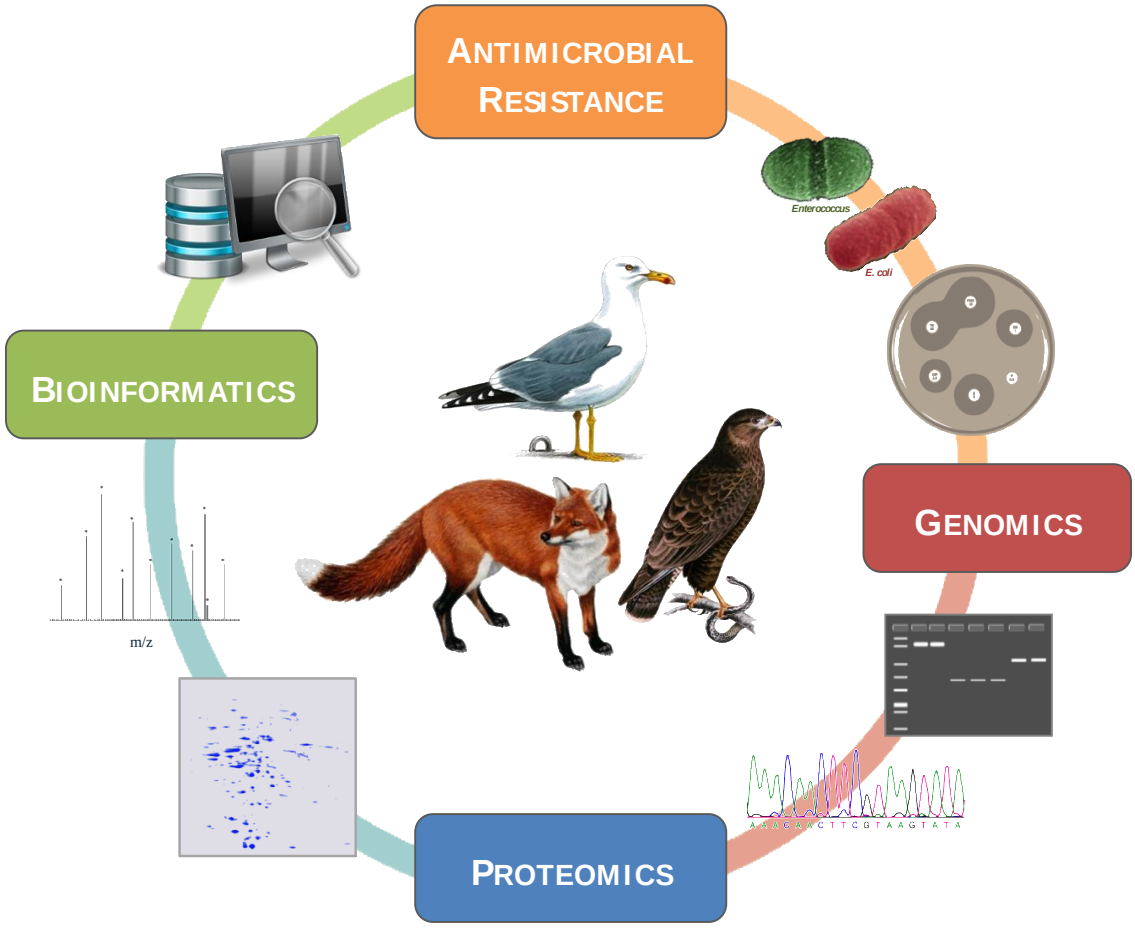
Les souches d'*E. coli* productrices de BLSE et d'entérocoques *van*A ont également été étudiés par une approche protéomique détaillée consistant à l'électrophorèse bidimensionnelle (E2D) et suivi de la technique de spectrométrie de masse à temps de vol pour la désorption-ionisation laser assistée par matrice (MALDI-TOF MS). Un total de 686 *spots* a été excisé des gels E2D de 3 souches d'*E. coli* productrices de BLSE et 4 souches de ERV, dont 562 protéines ont été identifiées

avec succès (81,9%) et caractérisées par le biais d'interrogation de base de données. Les protéines identifiées ont été associées à différentes fonctions biologiques comme la glycolyse, le transport, la biosynthèse, la résistance aux antimicrobiens, entre autres. Les résultats ont révélé aussi la présence chez les souches d'*E. coli* et d'entérocoques des protéines impliquées dans la réponse au stress, comme les protéines chaperons DnaK, WrbA, GroEL, entre autres. En outre, la protéine VanA (MM: 37419,10938 / PI: 5,79), qui empêche la liaison de la vancomycine/teicoplanine a été identifiée suite à l'analyse du protéome complet de souches d'entérocoques *van*A. Cette technique a révélé des profils protéiques des bactéries résistantes ainsi qu'en réponse à diverses conditions de stress, ce qui a permis une meilleure compréhension des mécanismes de résistance correspondants. Les analyses protéomiques quantitatives au niveau de protéines spécifiques ou subproteomes dans des souches d' ERV et d'*E. coli* productrices de BLSE fourniront de nouvelles connaissances fonctionnelles dans les mécanismes de résistance aux antimicrobiens facilitant ainsi l'identification de marqueurs diagnostiques ou pronostiques des maladies ainsi que la découverte de cibles thérapeutiques.

Cette thèse discute la prévalence et la propagation de la résistance aux antimicrobiens chez des bactéries indicatrices tels qu'*E. coli* et *Enterococcus* spp. aux niveaux des animaux sauvages, et prouvant ainsi que la faune sauvage pourrait représenter un important réservoir de bactéries résistantes et de gènes de résistance. En outre, cette étude a également révélé la présence de souches portant des gènes de virulence, appartenant aux clones à haut risque infectieux, en plus des préoccupations de santé publique découlant de la propagation d' ERV et d'*E. coli* productrices de BLSE dans des écosystèmes naturels.

MOTS-CLES: Animaux sauvages, entérocoques résistants à la vancomycine, *Escherichia coli* productrices de β-lactamases à spectre élargi, génomique, protéomique, résistance antimicrobienne.

| GRAPHICAL ABSTRACT



| LIST OF ABREVIATIONS

A

aac	Aminoglycoside acetyltransferase gene
aac(6')-Ie-*aph*(2'')-Ia	Gene encoding a bifunctional enzyme
aad	Aminoglycoside adenylyltransferase gene
ABC transporter	ATP-binding cassette transporter
ace	Accessory colonization factor gene
ACN	Acetonitrile
aer	Aerobactin gene
agg	Aggregation substance gene
AMC	Amoxicillin+clavulanic acid
AMK	Amikacin
AMP	Ampicillin
AMPs	Antimicrobial peptides
ant(6)	Aminoglycoside nucleotidyltransferase gene
APEC	Avian pathogenic *Escherichia coli*
aph(3')	Aminoglycoside 3'-phosphotransferase gene
APS	Ammonium persulfate
AT	Autotransporters
ATM	Aztreonam
ATP	Adenosine triphosphate

B

BHI	Brain heart infusion
bp	Base pair

C

ca.	Circa
cat	Chloramphenicol acetyltransferase gene
CAZ	Ceftazidime
CBB	Coomassie brilliant blue
CC	Clonal complex
CHL	Chloramphenicol
*chu*A	Receptor gene encoding outer membrane
CIP	Ciprofloxacin
cm	Centimetre
*cml*A	Nonenzymatic chloramphenicol resistance gene
*cnf*1	Cytotoxic necrotizing factor 1 gene
CRATAS	Center of collecting, welcome and handling of wild animals
CTX	Cefotaxime
cylL_SL_LABM	Cyl operon genes

D

D-Lac	D-lactate
D-Ser	D-serine
Da	Dalton
DAEC	Diffusely adherent *Escherichia coli*
ddl	D-alanine-D-alanine ligase
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol

E

E. avium	*Enterococcus avium*
E. casseliflavus	*Enterococcus casseliflavus*
E. coli	*Escherichia coli*
E. durans	*Enterococcus durans*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
E. gallinarum	*Enterococcus gallinarum*
E. hirae	*Enterococcus hirae*
E. mundtii	*Enterococcus mundtii*
E. raffinosus	*Enterococcus raffinosus*
EAEC	Enteroaggregative *Escherichia coli*
EARSS/EARSNet	European Antimicrobial Resistance Surveillance System/network
Ed	*Enterococcus durans*
Ef	*Enterococcus faecium*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
erm	Erythromycin ribosomal methylase gene
ERY	Erythromycin
ESBL	Extended-spectrum beta-lactamase
ESI	Electrospray ionization
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
esp	Extracellular surface protein gene
EST	Expressed sequence tags
ETEC	Enterotoxigenic *Escherichia coli*
EU	European union
ExPEC	Extraintestinal pathogenic *Escherichia coli*

F

FA	Formic acid
*fim*A	Type-1 fimbrial gene
FOX	Cefoxitin
fsr	Regulator of the expression of *gel*E gene

G

g	Gram
*gel*E	Gene of gelatinase
GEN	Gentamicin
*gyr*A	Gene for DNA gyrase

H

h	Hour
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
HSP	Heat shock protein
hyl	Hyaluronidase gene

I

ICN	Institute for nature conservation
IEF	Isoelectric focusing

IEF x SDS-PAGE	Isoelectric focusing x Sodium dodecyl sulphate polyacrilamide gel electrophoresis
IMP	Imipenem
IntI	Integron integrase gene
IPG	Immobilized pH gradient
IS	Insertion sequence

K

KAN	Kanamycin
Kbp	Kilobase pair
V or kV	Volt or kilovolt

L

LC	Liquid chromatography
LPS	Lipopolysaccharide

M

M	Molar
mA	Milliampere
MALDI	Matrix-assisted laser desorption ionization
MALDI-TOF/MS	Matrix assisted laser desorption ionization - time of flight mass spectrometry
MALDI-TOF/TOF MS	Matrix-assisted laser desorption/ionization time of flight/time of flight mass spectrometry
Mb	Megabit
MBGD	Microbial genome database
MDR	Multiple drug resistance
mg	Milligram
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
mM	Millimolar
mm	Millimetre
MNEC	Meningitis-associated *Escherichia coli*
Mr	Relative molecular mass
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification technology
Mw	Molecular weight

N

NT	Not tested
NaCl	Sodium chloride
NAL	Nalidixic acid
NL	Non Linear

O

ºC	Degree celsius
OM	Outer membrane

Omp	Outer membrane protein
ORF	Open reading frame

P

PAI	Pathogenicity island
*pap*C	P fimbriae PapC gene
*pap*GIII	P fimbrial adhesin PapG gene
*par*C	Gene for topoisomerase IV gene
PBP	Penicillin-binding-protein
pbp	Penicillin-binding-protein gene
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
pH	Potential of hydrogen
pI	Isoelectric point
PMF	Peptide mass fingerprinting
ppm	Parts per million
PTM	Post-translational modification

Q

QD	Quinupristin-dalfopristin
Qnr	Quinolone resistance

R

RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
rRNA	Ribossomal ribonucleic acid

S

s	Second
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMX	Sulfamethoxazole
ST	Sequence type
STR	Streptomycin
stx	Shiga toxin gene
sul	Sulphonamide dihydropteroate synthase gene
SXT	Sulfamethoxazole-trimethoprim

T

TCA	Trichloroacetic acid
TEC	Teicoplanin
TET	Tetracycline
TFA	Trifluoroacetic acid
TMP	Trimethoprim
Tn	Conjugative transposon
TOB	Tobramycin
TOF	Time of flight
Tris	Hydroxymethyl aminomethane
tRNA	Transfer ribonucleic acid
*tsp*E4.C2	DNA fragment

U

UPEC	Uropathogenic *Escherichia coli*
US	United States
UTIs	Urinary tract infections
UV	Ultraviolet

V

VAN	Vancomycin
V*an*	Gene conferring resistance to vancomycin and/or teicoplanin
vat	Streptogramin acetyltransferase gene
VF	Virulence factor
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*

W

WCP	Whole-cell protein
WHO	World Health Organization

Y

*yja*A	Gene with no known function

%	Percentage
1-DE	Monodimensional electrophoresis
2-DE	Bidimensional electrophoresis
μg	Microgram
μL	Microliter

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I.1. The genetic and evolutionary frontier of antimicrobial-resistant bacterial colonization and its implications for wildlife conservation

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| Manuscript under revision

I.1.1. Abstract

Given the significant spatial and temporal heterogeneity in antimicrobial resistance distribution and the factors that affect its evolution, dissemination and persistence, it is important to highlight that antimicrobial resistance must be viewed as an ecological problem. Monitoring the resistance prevalence of an indicator bacteria such as *Escherichia coli* and enterococci in wild animals makes it possible to show that wildlife has the potential to serve as an environmental reservoir and melting pot of bacterial resistance. These researchers address the issue of antimicrobial-resistant microorganism proliferation in the environment and the related potential human health and environmental impact.

I.1.2. Wildlife conservation in Portugal

In Portugal, the nature conservation policy acquired importance in the 70s, when the Law no. 9/70 of June 19th regarding the establishment of Protected Areas was delivered. After April 25th 1974, with the Decree-Law no. 550/75 of September 30th, the Environment State Secretariat was created, together with the National Park, Reserve, and Landscape Heritage Service, a body with legal competency, administrative and financial autonomy, that further in the 80s, with the Decree-Law no. 49/83 of January 31st, would become the National Park, Reserve, and Nature Conservation Service. In 1993, with the Decree-Law no. 19/93 of 23rd January, the new legal regime for the classification of the Protected Areas was elaborated and the Institute for Nature Conservation (ICN) was created with the Decree-Law no. 193/93 of May 24th (ICNB, 2012). This is a division of the Environment, Territory, and Regional Development Ministry that focuses on the study and identification of endangered habitats and species, in addition to the management of protected area (ICNB, 2012).

Portugal is one of the European countries with the highest diversity of organisms and farming systems, which at the same time, is at most risk of losing this diversity (MEA, 2004). Portugal has a number of very diverse fauna and flora in relation to its size, and is considered one of the 25 biodiversity hotspots of the world (MEA, 2004).

Due to political and economic circumstances, the Portuguese society was predominantly rural until around twenty years ago (ICNB, 2012). Land use changes have produced modifications in the Portuguese landscape, ecosystems and environment. Coastal zones are a combination of complex ecological systems and intense human occupation. These areas are subjected to constant pressures, as they are the focus of increasingly intensive urbanization, tourism and countless leisure activities leading to habitat loss. During the last 20 years, the widening of roads has increased considerably and this has had an impact on all areas with protection status, be it ecological reserves, reserves of agriculture, protected areas, Natura 2000 sites or areas of water in the public domain (EEA, 2012).

Wild animals are also adversely affected by habitat change caused by increasing pressure from agribusiness practices, but also by changes in depopulation and therefore land use. Intensive agriculture, monoculture tree plantations, continued urban expansion, enlargement of the road network and excessive hunting also affect the survival of certain species (EEA, 2012).

Usually, wildlife is not exposed to clinical antimicrobial agents but can acquire antimicrobial resistant bacteria through contact with humans, animals and the environment, where water polluted with faeces appears to be the most significant vector of contamination. The incidence of commensal and pathogenic bacteria in faecal contaminations can be expected to be a connection between the environment and settings with regular or even constant antimicrobial pressure (aquaculture, livestock farming, human, and veterinary clinical settings), resulting in a constant release of antimicrobial-resistant human and animal bacteria into the environment through wastewater or manure (Martinez, 2009). Additionally, the detection of antimicrobial-resistant bacteria in aquatic environments

affected by human and animal wastewater and soil provides evidence for this hypothesis (Kummerer & Henninger, 2003). In this context the common use of antimicrobials in aquaculture is also of utmost importance due to possible direct influences on wild animals (Smith, 2008). As intestinal bacteria like *Escherichia coli* and enterococci can be easily disseminated in different ecosystems through water, they are intensively used as indicator species for faecal pollution (Guenther et al., 2011).

I.1.3. *E. coli* and enterococci as ubiquitous bacteria

I.1.3.1. Biology and description of E. coli and enterococci

E. coli, first described in 1885, is a Gram-negative straight rod, catalase-positive and non-sporulating. It is a facultative anaerobic chemoorganotroph capable of both respiratory and fermentative metabolism (Blattner et al., 1997). *E. coli* can survive on a wide variety of substrates. It uses mixed-acid fermentation in anaerobic conditions, producing acetate, carbon dioxide, ethanol, lactate and succinate. Since fermentation pathways yield very little energy, this is generally a last resort metabolic process (Madigan et al., 2006).

E. coli is a common inhabitant of the intestinal tract of animals and humans (Sørum & Sunde, 2001; Tannock, 1995). *E. coli* and related bacteria constitute about 0.1% of gut microbiota (Eckburg et al., 2005) and this species can be easily spread in different ecosystems through water, soil, food, and others. Because it can transit in water and sediment, it is regularly used as an indicator of faecal pollution of water; using intuitive calculations, it has been estimated that half of the *E. coli* population inhabits in these secondary habitats (Savageau, 1983).

E. coli is the most widely studied prokaryotic model organism. While pathogenic strains have been significantly studied, few reports have focused on commensal strains, resulting in a bias towards pathogenic strains in the data sets (Tenaillon et al., 2010).

The reference strain *Escherichia coli* K-12 became a favourite for geneticists and molecular biologists who discovered and elaborated fundamental genetic and biochemical processes by studying this organism. In the wild, the size of *E. coli* total population has been estimated to be 10^{20}, and it has the characteristic of being both a widespread gut commensal of vertebrates and a versatile pathogen (Kosek et al., 2003; Russo & Johnson, 2003).

With its large range of pathologies, *E. coli* is a major cause of human morbidity and mortality around the world. Each year *E. coli* kills more than two million humans due to infant diarrhea (Kotloff et al., 1999) and extraintestinal infections (mainly septicaemia derived from urinary tract infection) (Russo & Johnson, 2003), and also causes about 150 million cases of uncomplicated cystitis (Russo & Johnson, 2003; Touchon et al., 2009).

Concerning enterococci, these bacteria were first described as a group by Thiercelin in 1899, and the genus *Enterococcus* was suggested later by Thiercelin and Jouhaud (1903) for Gram-positive diplococci of intestinal origin. Enterococci fit within the general definition of lactic acid bacteria and modern classification methods resulted in the transfer of some members of the genus *Streptococcus* to the new genus *Enterococcus* (Franz et al., 2003).

Therefore, enterococci are Gram-positive, catalase-negative, gamma-hemoltyic, non-spore-forming, facultative anaerobic bacteria (Fisher & Phillips, 2009). They usually inhabit the gastrointestinal tract of humans being also isolated from environmental and animal sources. They are able to survive a range of stresses and hostile environments, including those of extreme temperature (5–65ºC), pH (4.5-10.0) and high NaCl concentration (Palmer et al., 2012). The capability of enterococci to withstand broad pH ranges is likely due to their membrane durability and impermeability to acid and alkali, while their resistance to temperature is due to membrane lipids and fatty acids (Fisher & Phillips, 2009). The extreme conditions by which enterococci can survive permit them to colonize a wide range of niches, which could have implications for their clinical importance (Vu & Carvalho, 2011).

The enterococci colonizes with lifestyles ranging from intestinal symbiont to environmental persister to multidrug-resistant nosocomial pathogen (Aarestrup et al., 2002; Malani et al., 2002; Palmer et al., 2012; Tannock & Cook, 2002), and became one of the most common nosocomial pathogens with a mortality rate of up to 61% (Lopes Mde et al., 2006). The genus *Enterococcus*, after different taxonomical allocations that have identified more than 40 different species (Santagati et al., 2012), only those from humans and animals have been investigated in detail. The most important species are the potential human pathogens *Enterococcus faecalis* and *E. faecium*, though *E. gallinarum* and *E. casseliflavus* have also been studied because they are inherently vancomycin-resistant and colonize the intestinal tract (Murray et al., 2009).

Moreover, enterococci have been used extensively over the last decade in the food industry as probiotics or as starter cultures due to their capability to produce bacteriocins (Foulquié Moreno et al., 2006). As these microorganisms are used for tracking faecal contamination, they constitute regulatory and industrial concern (Fisher & Phillips, 2009).

I.1.3.2. Role in disease

Among the intestinal pathogenic *E. coli*, there are six well-described categories: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). Urinary tract infections (UTIs) are the most common extraintestinal *E. coli* infections and are caused by uropathogenic *E. coli* (UPEC). An increasingly common cause of extraintestinal

infections is the pathotype responsible for meningitis and sepsis – meningitis-associated *E. coli* (MNEC). The *E. coli* pathotypes implicated in extraintestinal infections have recently been named ExPEC, EPEC, EHEC and ETEC; and can also cause disease in animals using many of the same virulence factors that are detected in human strains and unique colonization factors that are not present in human strains. A further animal pathotype, known as avian pathogenic *E. coli* (APEC), causes extraintestinal infections – principally respiratory infections, pericarditis, and septicaemia of poultry (Kaper et al., 2004). In order to treat these infections, β-lactam antimicrobials are significantly used in human and veterinary medicine. In the last decade a variety β-lactamases emerged in Gram-negative bacteria, resulting in reduced susceptibility to broad-spectrum β-lactams (Faure et al., 2009).

Regarding to enterococci, they are considered emerging pathogens of humans and have become of major importance in community- and hospital-acquired infections such as endocarditis, bacteraemia, urinary tract, neonatal, central nervous system, intra-abdominal and pelvic infections. They are commonly source of infections in severely injured and immunocompromised patients who undergo prolonged and intensive antimicrobial therapy (Maki & Agger, 1988; Murray, 1990).

Within the last two decades enterococci became prominent as important hospital-acquired pathogens. Isolates of *E. faecalis* and *E. faecium* are the third- to fourth-most prevalent nosocomial pathogens worldwide (Werner et al., 2008). The first vancomycin-resistant enterococci (VRE) strains, *E. faecium* and *E. faecalis*, were isolated in 1986 in France (Leclercq et al., 1988) and in the United Kingdom (Uttley et al., 1989), and since then they have been found in many other countries (Bell et al., 1998; McGregor & Young, 2000; Suppola et al., 1999). In some countries, VRE may drastically contribute to enterococcal populations circulating in hospitals (Kawalec et al., 2001).

During the last years, a decrease of *E. faecium* resistant to vancomycin was observed in Portugal as in other geographical regions in Europe; efforts to control glycopeptide resistance seem to be successful and resulting in stabilisation or continuous decrease (EARS-Net results, 2011). Moreover, *E. faecium* have the unique ability of acquiring high-level resistance to aminoglycosides, ampicillin and vancomycin, the most efficient antienterococcal drugs (Amyes, 2007). Being part of the gastrointestinal flora, the enterococci are in a unique condition to receive resistance genes from other commensals, but also transfer these to other and more pathogenic bacteria located in the gastrointestinal tract (Engel et al., 1980). This highlights the clinical importance of enterococci as a reservoir for antimicrobial resistance determinants.

In this sense, it is essential to interpret the evolutionary and ecological forces that influence in the population structure of the commensal strains to fully understand the antimicrobial resistance and virulence of pathogenic strains. Certainly, the selective pressures in the habitats of commensal strains may coincidentally promote the emergence of antimicrobial resistance and virulence factors, rendering commensal strains reservoirs of virulent and resistant strains (Tenaillon et al., 2010).

I.1.4. Antimicrobial resistance pollution in wildlife

An important difficulty in evaluating the causal relationship between antimicrobial use and resistance is the confounding influence of geography: the co-localization of resistant bacterial species with antimicrobial use does not essentially involve causation and could represent the presence of environmental conditions and factors that have independently contributed to the incidence of resistance (Singer et al., 2006).

The collection of all antimicrobial resistance genes and their precursors in pathogenic and non-pathogenic bacteria and also in antimicrobial producing-organisms is referred as the antimicrobial resistome, a concept that has been advanced to serve as a framework for understanding the ecology of resistance on a global scale (Wright, 2010).

The expression of the multidrug-resistance phenotype in *E. coli* and enterococci isolates from wildlife is not the consequence of the nearby use of antimicrobials in natural environments, but is due to a distant use that had caused a multiresistant organism to evolve in the first place which consequently spread to different ecological niches (O'Brien, 2002).

Despite the commensal character of *E. coli* and enterococci, they are commonly involved in animal and human infections that implicate the use of antimicrobials, which increases public health preoccupations to the list of implications that arise from the spread of Extended-Spectrum Beta-Lactamase producing *E. coli* (ESBL-*E. coli*) and VRE into wildlife. Furthermore, the increasing frequency of community-acquired ESBL-*E. coli* and VRE infections and the occurrence in livestock farming has been observed recently, suggesting a successful transmission as well as persistence of ESBL-*E. coli* and VRE *st*rains outside hospital settings. An additional parallel global phenomenon is the spread of ESBL-*E. coli* and VRE into the environment beyond human and domesticated animal populations, and this appears to be directly induced by antimicrobial practice (Guenther et al., 2011). This might be a significant cause of the community-onset of ESBL-*E. coli* and VRE infections but can result (i) in an involvement of wildlife in ESBL-*E. coli* and VRE spread and transmission into fragile environmental niches, (ii) in subsequent colonization of wild animal populations which can turn into an infectious source or even a reservoir of ESBL-*E. coli* and VRE, (iii) in new putative infection cycles between wildlife, domesticated animals and humans, and (iv) in difficulties of wildlife medical treatment (Guenther et al., 2011).

Monitoring the prevalence of resistance in indicator bacteria such as faecal *E. coli* and enterococci in different populations (animals, patients and healthy humans) makes it possible to compare the prevalence of resistance and to detect transfer of resistant bacteria or resistance genes from animals to humans and vice versa (Martel et al., 2001). Just recently, there has been increasing interest in resistant bacteria and resistance genes isolated from wild animals (Allen et al., 2011).

The degree of colonization varies a lot between different animal species (Gordon & Cowling, 2003). Therefore, ESBL-*E. coli* and VRE prevalence is clearly influenced by sampling

schemes, by geographic regions, by host spectrum of these bacteria and by the degree of synanthropic behaviour shown by host species (Allen et al., 2010). It is important also to take in consideration the limitations that occur interpreting these results. For instance, ESBL-*E. coli* prevalence in different Portuguese geographical areas ranged from 0.5% in birds of the remote Azores islands in the Atlantic Ocean (Silva et al., 2011) to 32% for birds of the Portugal's Northern Portuguese coast (Simões et al., 2010). A lower prevalence of ESBL-*E. coli* was also observed (0.8%) in glaucous-winged gulls of Kamchatka peninsula in Russia (Hernandez et al., 2010). These findings suggest that wild animals living in urban areas are more susceptible to carry ESBL-*E. coli* than those living in remote areas.

Due to their diversity in ecological niches and their ease in picking up human and environmental bacteria, wild birds might act as mirrors of human activities. Within the heterogeneous class of birds, two groups seem to be in the focus of ESBL-*E. coli* and VRE carriage in wildlife: birds of prey (Costa et al., 2006; Pinto et al., 2010; Radhouani et al., 2010a; Radhouani et al., 2010b; Silva et al., 2011) and waterfowl/water related species (Bonnedahl et al., 2009; Dolejska et al., 2009; Garmyn et al., 2011; Guenther et al., 2010a; Hernandez et al., 2010; Literak et al., 2010a; Poeta et al., 2008; Radhouani et al., 2010c; Simões et al., 2010). Although wild birds, such as birds of prey, have only rare contact with antimicrobial agents, in disagreement with the existence of direct selective pressure, they can be contaminated or colonized by resistant bacteria. Water contact and acquisition via food seem to be major aspects of transmission of resistant bacteria of human or veterinary origin to wild animals (Cole et al., 2005). In the other hand, wild birds such as seagulls are often opportunistic marine feeders along the shoreline or offshore, but also eat the food sources provided by humans, especially garbage. Migrating birds that travel long distance seem to act as transporters, or as reservoirs, of resistant bacteria and may consequently have a significant epidemiological role in the dissemination of resistance, as well as being mirrors of the spectrum of pathogenic microorganisms present in humans (Radhouani et al., 2010c). Reports on marine fish showed the presence of ESBL-*E. coli* (Sousa et al., 2011) and VRE (Barros et al., 2012) in gilthead seabream, indicating a dissemination of ESBL-*E. coli* and VRE into the Atlantic ocean. Moreover, it has previously been demonstrated that seagulls shared strains with isolates cultured from wastewater treatment plants and landfills (Nelson et al., 2008). This highlights the possibility of bacterial exchange between human sewage and birds.

Another important host of these bacteria appears to be a group of rodents, with reports on ESBL-*E. coli* and VRE isolates from different continents (Guenther et al., 2010a; Ho et al., 2011; Mallon et al., 2002). This synantropic species can easily pickup human waste and frequently interacts with human faeces in the sewage system in urban environments and can therefore easily acquire multiresistant bacteria. Remarkably, wild boars have also been described as hosts of these bacteria in Europe, which might expose their omnivorous feeding behaviour (Literak et al., 2010b; Poeta et al., 2007; Poeta et al., 2009). Recent studies revealed the presence of ESBL-producing

E. coli (Gonçalves et al., 2012a; Gonçalves et al., 2012b) and VRE isolates (Gonçalves et al., 2011) in Iberian wolf and/or Iberian lynx. The incidence of ESBL-*E. coli* (Radhouani et al., 2012a) and VRE (Radhouani et al., 2011a) in red foxes may be due their diet as these wild animals usually hunt wild rabbits, small rodents and birds. It is important to point out that some studies reported the presence of antimicrobial resistant isolates in wild rabbits (Silva et al., 2010) and wild rodents (Guenther et al., 2010b). Foxes are on top of the food chain, perhaps accumulating multiresistant bacteria from their prey (Grobbel et al., 2012). All these evidences may contribute in the acquisition and spread of antimicrobial resistant bacteria even in the absence of direct antimicrobial pressure.

These wild animals act as reservoirs of resistance genes and they could spread resistant bacteria throughout the wild environment. These researchers address the issue of antimicrobial-resistant microorganism proliferation in the environment and the related potential human health and environmental impact.

The level of resistant bacteria detected in wild animals seems to relate well with the degree of association with human activity (Skurnik et al., 2006). In fact, human density, natural preservation state, livestock or the reserve of an area may be significant criteria for the proliferation of antimicrobial-resistant bacteria (Allen et al., 2010). Nevertheless, several studies report the occurrence of multidrug-resistant bacteria in remote places or preservation areas therefore underlining the complexity of the spread of antimicrobial resistance in wild animals. These discoveries propose, on one hand, an influence of migratory behaviour of wild birds into remote areas, or on the other hand the omnipresence of human influence in various ecological niches of the planet via human faeces (Guenther et al., 2011). Different reports showed that areas with high livestock and human density and an assumable frequent interaction of wildlife with human influenced habitats of any kind (livestock farms, landfills, sewage systems, or wastewater treatment facilities) result in a higher risk for wildlife to acquire antimicrobial-resistant bacteria (Allen et al., 2010).

I.1.5. Genome plasticity as paradigm of microbial genome evolution

Living organisms are defined by the genes they possess; and control of this gene set expression, both temporally and in response to the environment, determines whether an organism can survive changing conditions and can compete for the resources it needs to reproduce. Bacteria are not an exception; changes to the genome will, in general, threaten the ability of the microbe to survive, but acquisition of new genes may enhance its chances of survival by allowing growth in a previously hostile environment. For example, acquisition of an antimicrobial resistance gene by a bacterial pathogen can allow it to thrive in the presence of an antimicrobial that would otherwise kill it; this may compromise clinical treatments. Many forces, chemical and genetic, can alter the genetic content of Deoxyribonucleic acid (DNA) by locally changing its nucleotide sequence.

Genomic researchers have discovered that bacterial genomes are dynamic entities that evolve through numerous processes, including intrachromosome genetic rearrangements, gene duplication, and gene loss or acquisition by lateral gene transfer (Lawrence, 2005).

E. coli has only one circular chromosome that has been entirely sequenced by lab researchers; with about 4,600 kilobase pairs (kbp), about 4,300 potential coding sequences, and only about 1,800 known *E. coli* proteins. Seventy percent of the chromosome is composed of single genes (monocistronic), and 6% is polycistronic. About 30% of the sequenced Open Reading Frames (ORFs) have unknown functions (Reed, 2005).

The complete sequence of the *E. coli* (K-12) genome was reported by Science in 1997. The genome consists of a single molecule of DNA containing 4,639,221 bp. These encode 4,288 proteins and 89 ribonucleic acids (RNAs). Many of the genes were already known and the function of many others can be deduced from the similarity to known genes (Blattner et al., 1997). Comparison with five other sequenced microbes shows ubiquitous as well as narrowly distributed gene families; many families of similar genes within *E. coli* are also evident. The main family of paralogous proteins contains 80 ATP-binding cassette (ABC) transporters. The genome as a whole is extremely organized with respect to the local direction of replication; guanines, oligonucleotides possibly linked to replication and recombination, and most genes are oriented. The genome also contains insertion sequence (IS) elements, phage remnants, and many other patches of unusual composition indicating genome plasticity through horizontal transfer (Blattner et al., 1997). As with all organisms, the *E. coli* genome represents a satisfactory compromise between economy and versatility (Schaechter, 2001).

The enormous plasticity of the enterococci genome represents one of the major challenges in the analysis of these bacteria. In both *E. faecalis* and *E. faecium*, acquired elements can account for up to 25% of the genome (Arias & Murray, 2012). Genomic research into enterococci started with the identification and sequencing of a 153-kb pathogenicity island (PAI) in *E. faecalis* strain MMH594 (Shankar et al., 2002). This high-level gentamicin-resistant enterococci strain had caused multiple life-threatening infections in the mid-1980s (van Schaik & Willems, 2010).

The first complete genome sequence of *E. faecalis* was determined for strain V583, which was the first vancomycin-resistant clinical *E. faecalis* isolate to be reported in the United States of America (USA) (Paulsen et al., 2003). The circular chromosome of V583 comprised 3,218,031 bp with an average G+C content of 37.5%. A total of 3,182 ORFs with an average size of 889 bp, 1760 of these ORFs exhibit similarity to known proteins. Moreover, among the protein coding genes were 221 of unknown function, 495 with conserved hypothetical functions and 706 with no database matches. Extraordinarily unique to this genome was the fact that over 25% of the genome consists of mobile and/or exogenously acquired DNA which included a number of conjugative and composite transposons, a pathogenicity island, integrated plasmid genes and phage regions, and a high number of insertion sequence elements (Tendolkar et al., 2003). Concerning virulence factors, researchers

have discovered a pathogenicity island on the genome of *E. faecalis*. In fact, this strain contains most of the PAI but lacks the *esp* and *cyl* genes because a 17-kb DNA fragment carrying these genes has been excised from the PAI. This region contains about 150 kbp long with a lower G+C content than the rest of the genome and encodes genes that help bacteria in host infection, including genes for a toxin such as *cyl*L_L and *cyl*L_S that punctures cell walls and genes for molecules such as *ace* that aid the adherence of *E. faecalis* to surfaces (Paulsen et al., 2003).

The first genome sequence of *E. faecium* strain TX0016 (the designation DO for this strain is also commonly used) was announced in the year 2000 (Fox, 2000). This strain was isolated in 1992 from a case of endocarditis in the USA (Arduino et al., 1994). The genome size for this strain was estimated to be around 2.9 Mb, slightly smaller than the 3.2 Mb genome of *E. faecalis* strain V583. The genome of *E. faecium* contains 2,928,706 bp in 300 contigs of 20 reads or greater, with an inferred 37.8% G+C content and 3,309 potential protein-coding genes (Tendolkar et al., 2003). The sequence of TX0016 has not yet been finished. Indeed, in its current assembly, genes encoding products that are essential for bacterial life (such as ribosomal proteins) are missing, indicating that the currently available assembly of the TX0016 genome sequence is incomplete. Therefore, *E. faecium* may be the only major nosocomial pathogen for which no complete genome sequence is publicly available, and this may have resulted in the relatively poor understanding of the fundamental biology and virulence-associated traits of this organism compared to *E. faecalis*. This evident predisposition to acquire mobile gene elements has definitely contributed to the rapid acquisition and dissemination of drug resistance of the enterococci. There is also evidence that enterococci act as a reservoir of drug resistance for other genera, especially as concerns vancomycin resistance (van Schaik & Willems, 2010)

During decades, some microbes such as *E. coli* and enterococci served as model organisms in genetics and molecular biology. The same advantages – ease of culture, rapid generations and large populations – that served those fields also make microorganisms perfectly suited to experimental evolution (Reid & Buckley, 2011).

I.1.6. Fundamental evolutionary processes

Microorganisms play an important role in the cycling of elements at a global scale, thus profoundly and directly affecting the environments, in which all of life evolves. While microorganisms affect the environment, the environment in turn also engenders evolutionary pressures on the microorganisms themselves (Reid & Buckley, 2011).

Recent advances in DNA sequencing, high-throughput technologies, and genetic manipulation systems have permitted empirical studies that directly characterize the molecular and genomic bases of evolution (Conrad et al., 2011; Wagner, 2008). This launched the challenge of

unraveling the genotype-phenotype connection, with implications not only for the investigation of evolution, but also physiology, disease risk, development and biodiversity (Reid & Buckley, 2011).

In microbial populations, evolutionary change is supplied by two sources of new variation: horizontal transfer of genetic material from other, sometimes distantly related species, and mutation. Though, in the short run, mutation is the primary source of new genetic variation driving evolutionary change in microbial populations. In an asexual microbial species, evolutionary changes will happen by the repeated selection of new clones carrying adaptively favourable mutations (Adams, 2004).

In fact, firstly, genomic sequencing can determine the complete set of mutations responsible for an advanced phenotype, and has led to the discovery that interactions between these mutations are very usual. Secondly, adaptive mutations commonly target regulatory mechanisms. Thirdly, principles of systems-level optimization cause the genetic changes seen in adaptive evolution, and with a systems-level understanding, these optimization principles can be harnessed for the purposes of metabolic engineering. Fourthly, mutant sub-populations of enhanced fitness invariably arise in growing populations, but their dynamics in the population are complicated due to factors such as natural selection, clonal interference, drift, and frequency-dependent selection (Barrick et al., 2009).

The combination of mutation and horizontal transfer has created the overall phylogenetic structure of *E. coli* and enterococci, resulted to currently recognized four main phylogenetic groups for *E. coli* and different species for enterococci; and their respective lineages. Thus, an appreciation of mutation and horizontal transfer as important evolutionary processes within bacteria, in general, contributes in understanding of their roles, distribution and mechanistic modes of behaviour (Johnson, 2002).

I.1.7. Phylogenetic history and genetic structure

A multilocus enzyme electrophoresis (MLEE)-based phenogram using 38 enzymes (Goullet & Picard, 1989; Selander et al., 1986) identified four main phylogenetic groups (A, B1, B2 and D) and two accessory groups (C and E) in *E. coli* (Herzer et al., 1990; Selander et al., 1987; Tenaillon et al., 2010). These phylogenetic groups were recovered using the 1,878 genes of the *Escherichia* spp. core genome and the 2.6 million nucleotides of the *E. coli* chromosomal backbone (Touchon et al., 2009), which permitted a robust phylogeny to be developed; the first split in the *E. coli* phylogenetic history leads to one branch including the strains of group B2 and a subgroup within D that we called group F75 and another branch containing the rest of the species (Touchon et al., 2009). The remaining strains of group D then appeared from this second branch, followed by group E (Tenaillon et al., 2010). Finally, groups A and B1 are sister groups whereas group B2 is included in an ancestral

branch (Jaureguy et al., 2008; Touchon et al., 2009). The B2 group reveals the highest diversity at both the nucleotide and the gene content level (Touchon et al., 2009), supporting its early occurrence in the species lineage and suggesting that it has subspecies status (Lescat et al., 2009).

In 2000, Clermont et al. described a triplex Polymerase chain reaction (PCR strategy to assign *E. coli* isolates quickly to one of these phylogroups. It sought three phylogenetic group markers, the *chu*A and *yja*A genes encoding hypothetical proteins and the TSPE4.C2 DNA sequences situated within a gene encoding putative lipase esterase, and groups were assigned based on different combinations of presence and/or absence of the three amplicons (Clermont et al., 2000).

Johnson and co-workers (Johnson et al., 2001) found that strains from phylogenetic groups B2 and D contained more virulence factors than strains from the phylogenetic groups A and B1. Usually, the extraintestinal pathogenic strains belong to groups B2 and D (Johnson & Stell, 2000; Picard et al., 1999), the commensal strains to groups A and B1 (Bingen et al., 1998), whereas the intestinal pathogenic strains belong to groups A, B1 and D (Pupo et al., 1997).

Nowadays, these phylogenetic groups differ in their ecological niches, life-history (Gordon & Cowling, 2003) and some characteristics, such as their ability to exploit different sugar sources, their antimicrobial-resistance profiles and their growth rate (Carlos et al., 2010). A recent survey (Walk et al., 2007) demonstrated that the majority of the *E. coli* strains that are able to persist in the environment belong to the B1 phylogenetic group (Carlos et al., 2010).

Various researchers analysed the distribution of the main phylogenetic groups among *E. coli* strains isolated from human and animal faeces; it was found that the relative abundance of phylogenetic groups among mammals is dependent on the host diet, body mass and climate (Carlos et al., 2010; Gordon & Cowling, 2003). Furthermore, other study analysing faecal strains isolated from birds, non-human mammals and humans, observed the prevalence of groups D and B1 in birds, A and B1 in non-human mammals, and A and B2 in humans. These different reports concluded that one of the main forces that shapes the genetic structure of *E. coli* populations among the hosts is domestication (Escobar-Paramo et al., 2006). Moreover, faeces from zoo animals were analysed and a prevalence of group B1 in herbivorous animals and a prevalence of group A in carnivorous and omnivorous animals were found (Baldy-Chudzik et al., 2008). Furthermore, domesticated animals have a decreased proportion of B2 strains than wild animals (from 30% in wild animals to 14% and 11% in farm and zoo animals, respectively) and an increased proportion of A strains (from 14% in wild animals to 27% and 26% in farm and zoo animals, respectively) (Tenaillon et al., 2010).

In Portugal, the prevalence of *E. coli* of groups A and B1 was observed in wild birds as seagulls (Poeta et al., 2008; Radhouani et al., 2009) and birds of prey (Pinto et al., 2010; Radhouani et al., 2010a; Radhouani et al., 2012b), but also in wild mammals as Iberian lynxes (Gonçalves et al., 2012a) and in fish as gilthead seabream (Sousa et al., 2011). The report conducted by Simões and co-workers (Simões et al., 2010) showed that 37% of all *E. coli* isolates belong to B2 or D phylogroup, a higher rate than previously reported (27% of all *E. coli*) (Poeta et al., 2008) (Figure I.1.1).

It is interesting to note that faecal samples from red foxes showed that the *E. coli* isolates from phylogenetic groups A and D were predominant. Similar results were observed in chickens and swine (Machado et al., 2008), in wild boars (Poeta et al., 2009) and in wild birds (Radhouani et al., 2010a; Radhouani et al., 2012b) in the same geographical area.

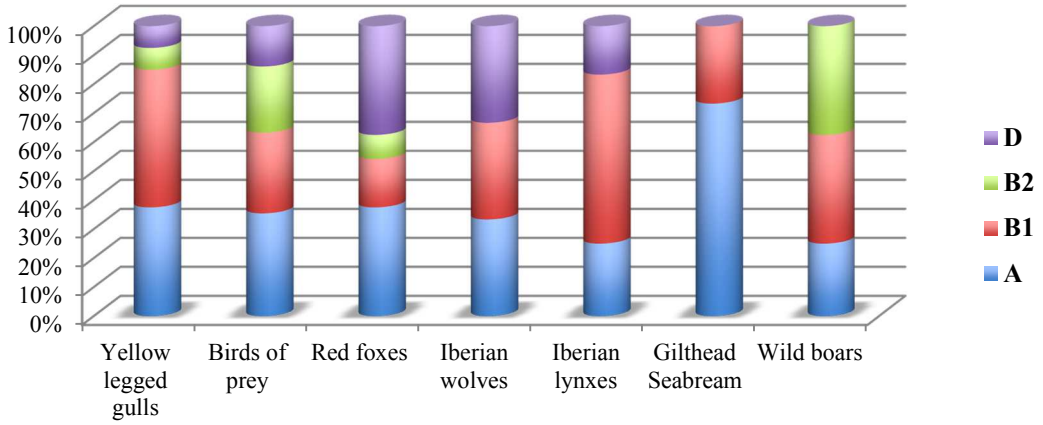

Figure I.1.1 | Phylogenetic group distributions of *E. coli* in wild animal from Portugal

Through the use of DNA hybridization and 16S rRNA sequencing, in 1984, it was established that the species *Streptococcus faecium* and *Streptococcus faecalis* were sufficiently distinct from the other streptococci to be designated another genus: *Enterococcus* (Fisher & Phillips, 2009). This approach also permitted the grouping of *Enterococcus* species. The *Enterococcus faecalis* species group includes *E. faecalis*, *E. haemoperoxidus* and *E. moraviensis* whilst the *Enterococcus faecium* species group includes *E. faecium*, *E. durans*, *E. hirae*, *E. mundtii*, *E. porcinus* and *E. villorum* (Fisher & Phillips, 2009). Concerning the *Enterococcus gallinarum* species group includes *E. casseliflavus* and *E. gallinarum*; the possibly linked *E. columbae* and *E. cecorum* represented a fourth group (Devriese & Pot, 1995; Franz et al., 1999; Williams et al., 1991). All other enterococci have been identified, i.e. *E. dispar*, *E. flavescens*, *E. saccharolyticus*, *E. sulfureus* and *E. seriolicida*, from individual lines of descent (Devriese & Pot, 1995; Devriese et al., 1993).

With the exception of *E. faecium* and *E. faecalis*, the enterococci are infrequently described to be involved in human pathogenesis (Devriese & Pot, 1995; Jett et al., 1994). Though, in some countries association of strains of *E. faecalis* and *E. faecium* with human disease has reached proportions of serious concern (Fisher & Phillips, 2009; Jett et al., 1994; Leclercq, 1997). Actually, the majority of hospital-derived isolates of *E. faecalis* cluster in two clonal complexes, CC2 and CC9 (Ruiz-Garbajosa et al., 2006; van Schaik & Willems, 2010), and the increased occurrence of *E. faecium* isolation world-wide is due to the presence of a polyclonal subpopulation, principally Multilocus Sequence Typing (MSLT) sequence type 17 (ST17), ST18, ST78 and ST192, which were previously designated as clonal complex CC17 (Willems et al., 2005; Willems & van Schaik, 2009).

Recent comparisons of available genome sequences support the concept of a hospital-associated clade that is genetically distinct from most commensal isolates from animals and humans (Galloway-Pena et al., 2012; van Schaik & Willems, 2010; Werner et al., 2011; Willems & van Schaik, 2009).

In Portugal, concerning wild animals, usually *E. faecium* and *E. faecalis* were found to be the predominant species in seagulls (Radhouani et al., 2011b; Radhouani et al., 2010c), birds of prey (Radhouani et al., 2010b; Radhouani et al., 2012b; Silva et al., 2011), wild rabbits (Figueiredo et al., 2009; Silva et al., 2010), partridges (Araújo, 2009) and gilthead seabream (Barros et al., 2012; Barros et al., 2011) faecal samples, among others, while *E. casseliflavus* and *E. gallinarum* were dominant in Iberian wolves and Iberian lynxes faecal samples (Gonçalves et al., 2011) (Figure I.1.2).

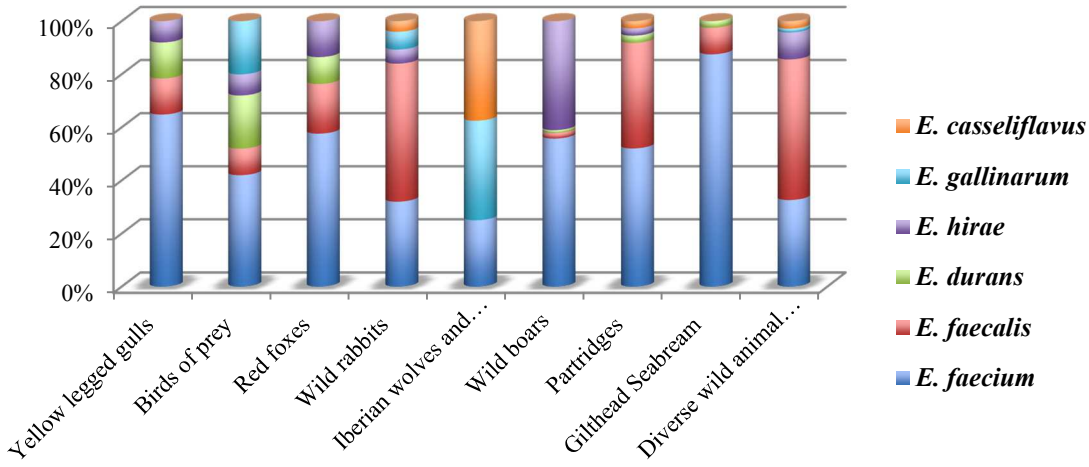

Figure I.1.2 | Distribution of *Enterococcus* species in wild animals from Portugal

Along with seagulls and birds of prey in Portugal, migratory Canada geese report showed also the occurrence of *E. faecium* and *E. faecalis* species. Furthermore, different reports found that all enterococci isolates detected in wild mammals (Mallon et al., 2002) and also in glaucous gulls (Drobni et al., 2009) were *E. faecium* species. Another study performed in Brazil, showed the occurrence of *E. faecalis* in nonhuman primate (capuchin monkeys and common marmoset) faecal samples (Xavier et al., 2010). In American bison faecal samples, *E. casseliflavus* was the predominant species recovered with 62.4%, followed by *E. faecalis* (16%).

With the arrival of next-generation sequencing (Mardis, 2008), it was quickly possible to investigate hundreds of strains to get a better knowledge at the whole-genome level the evolutionary processes acting in populations (Liti et al., 2009; MacLean et al., 2009; Schacherer et al., 2009), opening the era of 'population genomics' (Tenaillon et al., 2010). Certainly further studies using new high throughput technologies are mandatory to completely understand the evolution of predominant clones and species in different hosts and environments (Santagati et al., 2012).

I.1.8. Diversity and complexity of bacterial niches

I.1.8.1. Virulence factors

Large-scale epidemiological studies provide insight into the diversity and complexity of *E. coli* and enterococci niches (Arias & Murray, 2012; Tenaillon et al., 2010). Thus, genome plasticity has contributed to the emergence of new virulence traits: some clusters of genes or genomic islands (including pathogenic islands) should be discovered only in a subset of strains and favoured in some specific environments. Furthermore, several alternative combinations of genes could promote similar adaptations to a given environment (Tenaillon et al., 2010).

The genome has several known or suspected roles in the evolution of pathogenic strains. Genome size is greater among pathogenic bacteria than among the nonpathogenic wild-type strain or laboratory strains, a consequence largely of the extra DNA contained in the multiple PAIs and other virulence factors (VF) present in the pathogenic strains (Johnson, 2002; Rode et al., 1999). The prevalence of VF genes is variable among commensal populations. On a global scale, the human microbiota is characterized by a higher prevalence of virulence genes than the microbiota of other organisms (Skurnik et al., 2008). In animals, the presence of virulence genes increases with body mass, which reveals the gut complexity of larger animal (Skurnik et al., 2008). Thus, virulence factors and their change in prevalence among hosts may reflect some local adaptation to commensal habitats rather than virulence *per se* (Tenaillon et al., 2010).

I.1.8.2. Intra-species interactions

Interactions between community members are required for community development and maintenance; and can also drive some diversification. To out-compete other clones, the production of colicins can represent a useful strategy in a structured environment (Chao & Levin, 1981). Colicins are the most expansively studied bacteriocins produced by *E. coli*. This could permit unadapted strains to colonize the gut and, hence, allow numerous clones to coexist in the long term. It may also promote diversification in a clone, as some strains may try to benefit from the production of the colicin but avoid paying the associated cost. Actually, the secretion of any enzyme or metabolite can be defined as an altruistic performance that can benefit some mutants or other strains lacking that component (Tenaillon et al., 2010).

In the other hand, the identification of PAIs is an important element in the evolution of bacterial pathogens through horizontal spread of virulence genes, similar to the horizontal transfer mediated by plasmids, bacteriophages and transposons (Tendolkar et al., 2003). The fact that these microorganisms are less virulent than others is not reassuring, since the acquirement of virulence genes by bacteria is possible (Leclercq, 1997). Further research is required to characterize molecular and cellular interactions between the host and enterococci which lead to intra-species genetic transfer and virulence factors in enterococci species (Giridhara Upadhyaya et al., 2009).

I.1.8.3. Antimicrobial resistance

Antimicrobial resistance is a worldwide problem in human and veterinary medicine. Commonly, it is usual that the major risk factor for the increase of this situation is an extensive use of antimicrobials that leads to the dissemination of resistant bacteria and resistance genes in animals and humans (van den Bogaard & Stobberingh, 2000). The appearance of multiresistant bacteria of human and veterinary origin is probably accompanied by co-contamination of the environment apparently leading to a great health concern (Grobbel et al., 2007). Bacteria may present resistance to antimicrobials under selective pressure, but they may also acquire resistance determinants without direct exposure to an antimicrobial through horizontally mobile elements including conjugative plasmids, integrons and transposons (Middleton & Ambrose, 2005). These mobile elements can simply transfer antimicrobial resistance genes from one bacterium to another (Coque et al., 2008). Antimicrobial agents exert a selective pressure not only on pathogenic, but also on commensal bacteria of the intestinal tract of humans and animals (van den Bogaard & Stobberingh, 2000). In a broader view, increasing evidence suggests that components such as integrons and their gene cassettes played significant roles in genome evolution and fluidity within the bacterial kingdom (Davies & Davies, 2010; Duriez et al., 2001).

In *E. coli*, the phylogenetic group A strains (Mammeri et al., 2009) and some group D strains (Deschamps et al., 2009) are especially permissive to the development of resistance to third-generation cephalosporins. Conversely, phylogenetic group B2 strains are less resistant than the remaining strains (Johnson et al., 1991; Johnson et al., 1994; Picard & Goullet, 1989), regardless of the molecular mechanism implicated in the acquisition of resistance, and have a lower prevalence of integrons in commensal *E. coli* strains from both human (Skurnik et al., 2005) and animal hosts (Skurnik et al., 2006). This could reveal the relative decrease of phylogenetic group B2 strains in domesticated animals in which antimicrobials are used considerably (Tenaillon et al., 2010).

The increase in the occurrence of nosocomial infections caused by enterococci in particular *E. faecium*, is at least partly due to the wide variety of intrinsic and acquired resistances characteristic of these species. Moreover, infections caused by other *Enterococcus* species (*E. faecalis*, *E. durans*, *E. mundtii*, *E. avium*, *E. raffinosus*, *E. gallinarum*, and *E. casseliflavus*) occasionally occur and warrant attention (Murray, 1990; Prakash et al., 2005). Enterococci are expert in acquiring and transferring elements that confer resistance to antimicrobials and they are also known to be intrinsically resistant to numerous antimicrobials. As a result, therapeutic alternatives for treatment of enterococcal infections are increasingly limited (Murray, 1990). Evolution of enterococci toward resistance to multiple antimicrobials is also a major cause of concern.

I.1.9. Antimicrobial resistance of bacteria: an example of evolution in action?

The strength of trillions upon trillions of microorganisms, combined with the ancient force of evolution by constant, insistent variation, will inevitably overpower the drugs. Their spectrum is selected to involve pathogenic bacteria and antimicrobials constantly select naturally resistant bacteria (American Academy of Microbiology, 2009). As bacteria quickly evolve to acquire resistance to the available antimicrobials, it is a constant race for scientists to develop effective strategies to combat infection and to reveal new therapeutic targets (Davies & Davies, 2010).

Moreover, antimicrobial resistance evolving and spreading among bacterial pathogens is a public health problem of increasing magnitude. Since the beginning of the antimicrobial era, the selective pressure caused by the use of antimicrobials in clinical, veterinary, husbandry and agricultural practices is considered the major factor responsible for the occurrence and spread of antimicrobial-resistant bacteria. The evolution of antimicrobial resistance in bacteria is related to the evolution of antimicrobial production. Though, resistance has also been discovered in the absence of antimicrobial exposure, as in bacteria from wildlife, raising an interest about the mechanisms of emergence and persistence of resistant strains under similar conditions, and the implications for resistance control strategies (Pallecchi et al., 2008). Monitoring antimicrobial resistance in wildlife from remote areas could also be a useful tool to evaluate the impact of anthropic pressure (Thaller et al., 2010).

Singer and co-workers support that in ecological studies of antimicrobial resistance, there has possibly been too much focus on resistant organisms and not enough on resistance genes. Due to the capability of bacteria to transfer resistance genes, even among distantly related bacteria, analyses of antimicrobial resistance emergence, dissemination and persistence might be better conducted at the gene level (Singer et al., 2006).

Until now, genomics-based investigation into *E. coli* and enterococci has focused on the identification of genes directly implicated in virulence. However, the fundamental physiology and response mechanisms to environmental conditions of *E. coli* and enterococci remained relatively poorly understood. This is a serious oversight because during infection the microbial fitness is an important reason in the success of any microbial pathogen. Further genome-wide reports aiming to define genes that are important during infection and colonization or exposure to antimicrobials will deliver significant data on relevant aspects of *E. coli* and enterococcal biology. This knowledge can consequently be useful for the development of novel treatment approaches to combat microbial infections.

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I.1.11. References

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I.2. Antimicrobial resistance: genomic insights of an old problem

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I.2.1. Abstract

More than half a century has passed since the first antimicrobials were introduced commercially. It did not take long for microbes to develop antimicrobial resistance, and widespread use of many antimicrobial drugs delivers ideal conditions for the spread of multidrug resistant organisms. Bacteria adopt intricate strategies to avoid the lethal effects of antimicrobials. The awareness of these mechanisms of resistance can help in the design of new drugs. Through genomics approaches, the potential for the emergence of resistance should garner newfound respect for the discovery of new agents so urgently wanted to treat infectious diseases.

I.2.2. Antimicrobial resistance as a global public health problem

I.2.2.1. History and emergence of antimicrobial resistance

Antimicrobials are described as substances produced by microorganisms (bacteria, fungi, actinomycetes), which suppress the proliferation of other (pathogenic) microorganisms and can possibly destroy these or substances derived from them. The term "antimicrobials" also include synthetic antibacterials such as the quinolones and sulfonamides, among others.

Antimicrobials are among the most important accomplishments of the 20th century. The appearance of antimicrobials transformed human and animal health systems by revolutionizing our weaponry in the war against infectious diseases, resulting in improved survivability for both humans and animals. The golden age of antimicrobial treatments was carried out by the discovery and subsequent development of penicillin from the filtrate of a fungus *Penicillium notatum* culture by Alexander Fleming in 1929.

Different antimicrobial agents have quite different chemical, physical and pharmacological properties, as well as different antibacterial spectra and mechanisms of action (Franco et al., 2009). Though, this health achievement was instantly failed by the subsequent realization that bacterial populations could rapidly modify themselves to resist antimicrobials, propagate these resistance traits, and even share resistance genes with other bacteria. Such capacities have seriously compromised the efficacy of antimicrobials in the war against microbes and warn of a future when antimicrobials may have very limited helpfulness to control bacterial infection (Spellberg et al., 2008).

Antimicrobial resistance is defined as the capability of a microorganism to survive and multiply in the presence of an antimicrobial agent that would usually inhibit or kill this specific kind of organism (James et al., 2012). Antimicrobial resistance is one of the several adaptive traits that resistant bacterial subpopulations may possess or acquire, enabling them to out-compete and out-survive their microbial neighbours and overcome host strategies aimed against them (James et al., 2012). The first reports of resistance appeared only one year after the initial introduction of penicillin. Likewise in 1959, the introduction of methicillin, the next-generation drug for the treatment of penicillin-resistant strains, was followed only two years later by the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) (Parker & Jevons, 1964). This statement repeats again and again as new antimicrobials are discovered and introduced into widespread use (James et al., 2012). Researchers showed today an alarming level at which antimicrobial resistance often develops and how quickly it spreads across the globe and among different species of bacteria. Moreover, as a result of sequential, cumulative acquisition of resistance traits against different antimicrobials, more bacterial pathogens with multiple-drug resistance are being described worldwide (James et al., 2012).

I.2.2.2. Causes of antimicrobial resistance

Antimicrobial resistance has become a major clinical and public health problem. Although it is clear that those antimicrobials are crucial in the selection of bacterial resistance, the spread of resistance genes and resistant bacteria also contributes to the alarming problem. Selection of resistant forms can occur during or after antimicrobial treatment; antimicrobial residues can be found in the environment for long periods of time after treatment (Figure I.2.1) (Levy & Marshall, 2004).

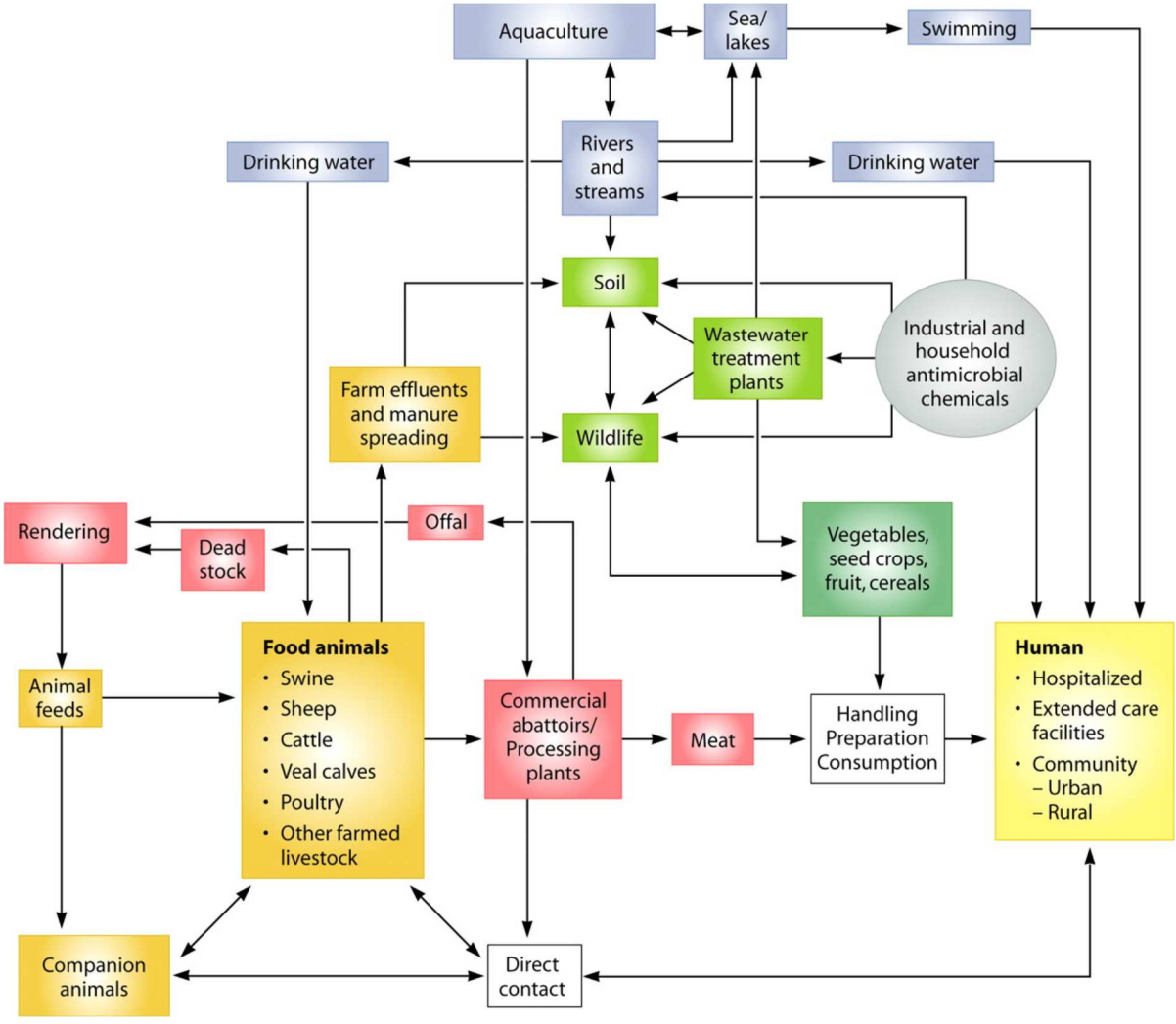

Figure I.2.1 | Dissemination of antibiotics and antibiotic resistance within agriculture, community, hospital, wastewater treatment, and associated environments

Selective pressure. A diversity of factors are responsible for antimicrobial resistance, however the selective pressure exerted by inappropriately used antimicrobials is possibly the most important. In the presence of an antimicrobial, microorganisms are either killed or, if they carry resistance genes, survive. These survivors will replicate, and their progeny will quickly become the dominant type throughout the microbial population (Albrich et al., 2004).

Mutation. Most mutations that lead to changes in amino acid sequences will be lost in the absence of selective pressures, as the resultant gene products will frequently function less efficiently than the original ones. In the presence of the antimicrobial (a selective pressure), the mutations that lead to resistance against the antimicrobial will be increased, as the bacterial clone with such mutations is the 'fittest to survive', while the others will be killed or inhibited by the antimicrobial (Woo et al., 2003).

Gene transfer. Horizontal gene transfer (HGT) has been responsible for the dissemination of numerous antimicrobial resistance determinants throughout diverse bacterial species. The rapid and broad dissemination of resistance determinants by HGT, and subsequent selection for resistance imposed by the use of antimicrobials, threatens to undermine the usefulness of antimicrobials (Barlow, 2009).

Societal pressure. The almost overwhelming complexity of factors influencing antimicrobial consumption includes cultural conceptions, diagnostic uncertainty, patient demands, economic incentives, the level of training among health staff and pharmacists, and advertising to prescribers, consumers and providers from the pharmaceutical industry (Cars & Nodberg, 2004).

Inappropriate use. Selection of resistant microorganisms is exacerbated by inappropriate use of antimicrobials. Sometimes, healthcare providers will prescribe antimicrobials inappropriately, wishing to placate an insistent patient who has a viral infection or an as-yet undiagnosed condition. Antimicrobials appear to be used not only in excess but also inappropriately and this accounts for 20% to 50 % of all antibiotics used (Bisht et al., 2009). The mechanisms behind this overuse are many and intricate. Studies from some developing countries show that several antimicrobials are generally prescribed at each consultation (Cars & Nodberg, 2004).

Inadequate diagnostics. Underuse, through lack of access to antimicrobials, inadequate dosing and poor adherence to therapy, may play a significant role in driving resistance as overuse. The use of broad-spectrum antimicrobial agents as a substitute for precise diagnostics or to enhance the likelihood of therapeutic success increases the rate of selection of resistant bacteria (Cars & Nodberg, 2004).

Hospital use. According to the World Health Organization (WHO), the combination of highly susceptible patients, intensive and prolonged antimicrobial use, and cross-infection has resulted in nosocomial infections with highly resistant bacterial pathogens. Resistant hospital-acquired infections are costly to control and extremely hard to eradicate (Allerberger et al., 2008).

Agricultural use. There is considerable debate surrounding the complex relationship between antimicrobial use in animals and the resistance problem in humans. The chronic use of subtherapeutic amounts of antimicrobials for growth promotion in food animals has been banned in the European Union, but it still be used in the United States. Despite their low-level application, the antimicrobials select determinants mediating high-level, clinically important resistance (Levy & Marshall, 2004). The transfer of enteric organisms may happen through the food chain or through

animal handlers. If the organisms are multidrug resistant, the occurrence of their resistance results essentially from use and overuse of antimicrobials in the animals. Generally, animal contributions to the resistance problem in human infections are minor but not insignificant; they have a major role if enteric organisms are involved (Levy & Marshall, 2004).

I.2.2.3. Implications of antimicrobial resistance

Throughout the selection pressure caused by antimicrobial use, a large reservoir of resistance genes has been generated. Nowadays, researchers show the impact of this situation on health. Due to resistant bacteria, failure of the preliminary antimicrobial treatment increases the risks of secondary complications and a fatal outcome, underscoring the clinical dilemma of empirical therapy and the prevailing lack of rapid diagnostic tests. A report about intensive care showed considerably higher mortality among patients that received inadequate empirical therapy, compared with those given adequate therapy (Cars & Nodberg, 2004). This is a vicious cycle, where increasing levels of resistance require the use of broader, more powerful antimicrobials in order to treat and to secure the survival of patients. Consequently, the use of these reserve antimicrobials intensifies the problem, as resistance develops and generates a situation where effective antimicrobials are lacking (Cars & Nodberg, 2004).

According to the WHO, approximately 25,000 patients die every year in the European Union due to resistant bacterial infection acquired in hospitals. Infections from resistant bacteria are a serious problem in health care settings, causing life-threatening infections in the blood stream, pneumonia, wound infections and others. Antimicrobial resistance increases the costs of treatment as a consequence of extended hospital stays, more expensive antimicrobial drug use and treatment (WHO, 2012).

Besides the clinical consequences of antimicrobial resistance, large societal costs are strictly associated. The most particular example is the cost of drug development, as new empirical treatments are required to combat resistant pathogens (Cars & Nodberg, 2004). In fact, resistance is costly and its worldwide economic impact is difficult to exactly quantify. In the European Union and Norway, the six most frequent resistant infections account roughly 2.5 million extra hospital days annually. The costs of infections were estimated at €1.5 billion each year, with more than €900 million related to hospital costs (DeWaal et al., 2011). In addition to this adverse scenario, pharmaceutical companies invest tremendously to push through the arduous process (Dickson & Gagnon, 2004), which might take up to 8-10 years, time during which multi-drug resistance may happen to the class of drug in development. Consequently, the new drug might become ineffective even before it is marketed. The economic and health costs of resistance, are serious in industrialised world, but often more severe in developing countries (Cars & Nodberg, 2004).

Universally, the antibiotic market generated sales of almost €32 billion in 2009, representing 46% of sales of anti-infective agents (including antiviral drugs and vaccines) and 5% of the global

pharmaceutical market (Hamad, 2010). Global demand for antibiotics is estimated to reach €34.1 billion in 2016. The market has been increasing with an average annual growth of 6.6% between 2005 and 2011. The market expansion is expected to slow to 4.6% in the coming years. Currently, the industry is dominated by some antibiotics (includes e.g. aminoglycoside antibiotics) which covers 79% of the demand while penicillins has 8%, erythromycin 7%, tetracyclines 4%, chloramphenicol 1% and streptomycins 1% share of the market (GR&DS, 2012).

One significant and usually forgotten aspect of antimicrobials and their resistance genes is that they evolved in non-clinical (natural) environments before the use of antimicrobials by humans. Antimicrobial compounds can currently be found in liquid waste at animal feedlots and fish-breeding locations, in lakes and ground-water supplies. Given that microorganisms essentially form the biosphere, learning the functional role of antimicrobials and their resistance components in nature has significant implications both for human health and from an ecological perspective. Furthermore, ecological niches outside the health care sector are changing, as bacteria formerly susceptible to antimicrobials develop resistance to them (Cars & Nodberg, 2004).

I.2.3. Mechanisms of antimicrobial action

Most antimicrobial agents used for the treatment of bacterial infections may be classified according to their principal mechanism of action. There are 4 major modes of action: (1) interference with cell wall synthesis; (2) inhibition of protein synthesis; (3) interference with nucleic acid synthesis (DNA gyrase and DNA-directed RNA polymerase); and (4) inhibition of a metabolic pathway (Figure I.2.2 and Table I.2.1) (Coates et al., 2002; Tenover, 2006).

I.2.3.1. Inhibition of cell wall synthesis

Antibacterial drugs that act by inhibiting bacterial cell wall synthesis include the β-lactams, such as penicillins, cephalosporins, carbapenems, monobactams and glycopeptides, including vancomycin and teicoplanin (McManus, 1997; Neu, 1992). β-Lactam agents inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for synthesis of the peptidoglycan layer (McManus, 1997). Vancomycin and teicoplanin also interfere with cell wall synthesis, but achieve so by binding to the terminal D-alanine residues of the nascent peptidoglycan chain, thus inhibiting the cross-linking steps required for stable cell wall synthesis (McManus, 1997).

I.2.3.2. Inhibition of protein synthesis

Aminoglycosides, macrolides, tetracyclines, chloramphenicol, streptogramins and oxazolidinones exert their antibacterial properties by inhibiting protein synthesis (McManus, 1997; Neu, 1992). Bacterial ribosomes differ in structure from their counterparts in eukaryotic cells.

Antibacterial agents take advantage of these differences to selectively inhibit bacterial growth. Macrolides, aminoglycosides, and tetracyclines bind to the 30S subunit of the ribosome, while chloramphenicol binds to the 50S subunit (Tenover, 2006).

I.2.3.3. Interference with nucleic acid synthesis

Fluoroquinolones produce their antibacterial effects by disrupting DNA synthesis and causing lethal double-strand DNA breaks during DNA replication, whereas sulfonamides and trimethoprim (TMP) block the pathway for folic acid synthesis, which finally inhibits DNA synthesis (Tenover, 2006).

I.2.3.4. Inhibition of a metabolic pathway

The usual antibacterial drug combination of TMP, a folic acid analogue, plus sulfamethoxazole (SMX) (a sulfonamide) inhibits two steps in the enzymatic pathway for bacterial folate synthesis. Disruption of bacterial membrane structure may be a fifth, though less well described, mechanism of action. It is postulated that polymyxins exert their inhibitory properties by increasing bacterial membrane permeability, causing leakage of bacterial contents. Apparently, the cyclic lipopeptide daptomycin inserts its lipid tail into the bacterial cell membrane, causing membrane depolarization and certain death of the bacterium (Tenover, 2006).

I.2.4. Mechanisms of antimicrobial resistance

Resistance mechanisms to avoid the toxic action of antimicrobials have been identified by researchers and described for all known antimicrobials actually available for clinical use in human and veterinary medicine. Five main molecular mechanisms of antimicrobial resistance have been characterized (Figure I.2.3 and Table I.2.2): 1) inactivation of the drug, 2) modification of the site of action (enzyme, ribosome, cell-wall precursor), 3) modification of the permeability of the cell wall, 4) overproduction of the target enzyme, and 5) the bypass of the inhibited steps (Coates et al., 2002).

Consequently, different agents engender resistance at different rates. Factors that determine whether resistance develops are complex and interdependent, and include: i) mechanisms of action, ii) whether the antimicrobial drug is a concentration or time-dependent killing agent, iii) the potency against the population of microorganism and iv) the magnitude and duration of the available serum concentration. It appears that once an antimicrobial agent is broadly used in the human population, resistance quickly develops, at least in some species of microbe (Amsden et al., 2000).

The initial phase of adaptation to antimicrobials includes acquiring resistance by one or more mutations that increase drug resistance through these mechanisms. All these mechanisms are related with fitness costs (for example, reduced maximal growth rate), and subsequent adaptation to

antimicrobials implicates fixing compensatory mutations that ameliorate this cost while preserving resistance. Bacterial antimicrobial resistance can be attained through intrinsic or acquired mechanisms (MacLean et al., 2010).

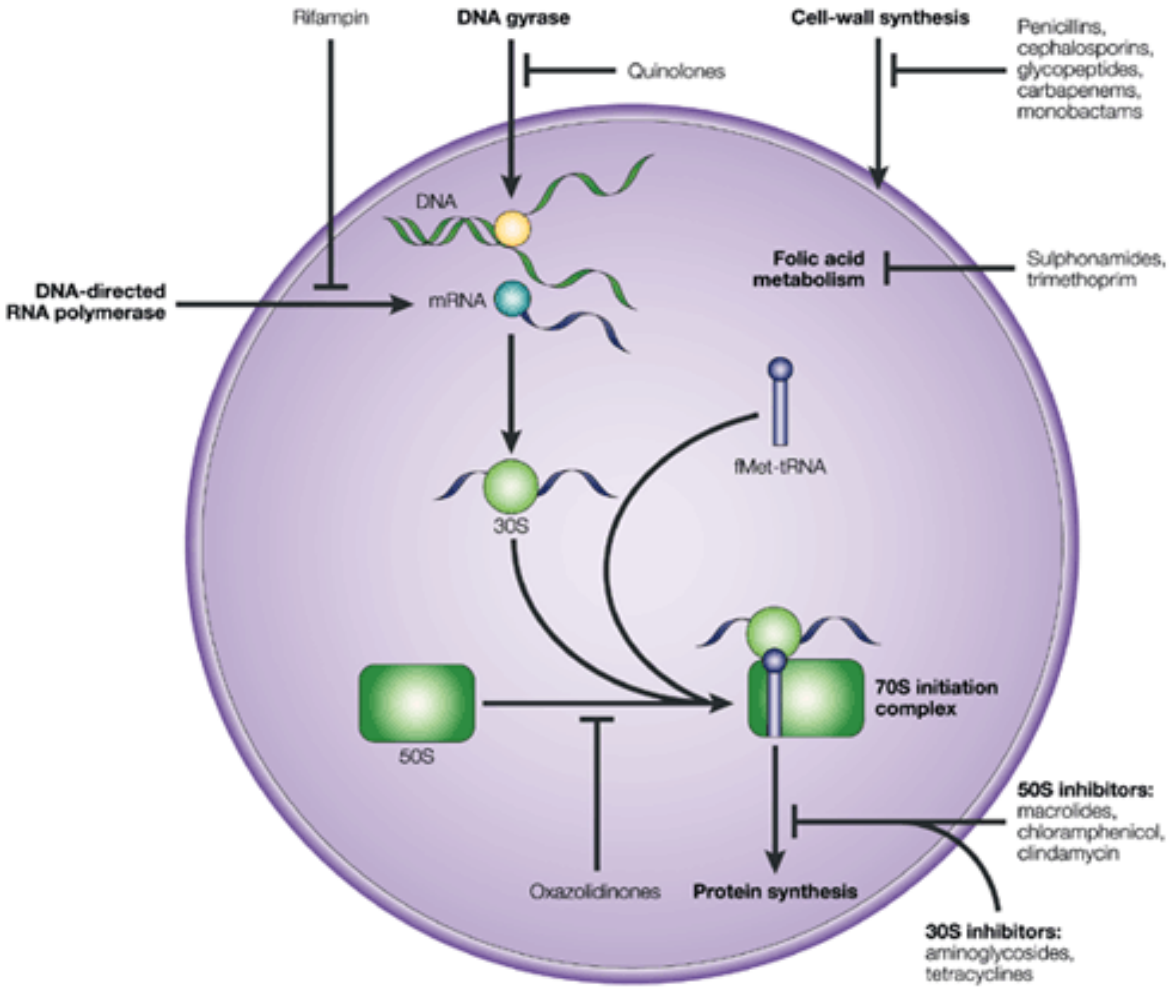

Figure I.2.2 | Mechanisms of antimicrobial action

(1) interference with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis (DNA gyrase and DNA-directed RNA polymerase), and (4) inhibition of a metabolic pathway. The figure shows the antimicrobial agents that are directed against each of these targets. In the case of protein synthesis, aminoglycosides and tetracyclines inhibit 30S RNA, and macrolides, chloramphenicol and clindamycin inhibit 50S RNA. mRNA, messenger RNA. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery] (The future challenges facing the development of new antimicrobial drugs, 1: 895-910), copyright (2002) **(**Coates et al., 2002**)**

Table I.2.1 | Antimicrobial agents and their mechanism of antimicrobial action

Antimicrobial class		Antimicrobial	Spectrum of activity	Primary target	Pathways affected
Fluoroquinolones	DNA synthesis inhibitor	Nalidixic acid, ciprofloxacin, levofloxacin, gemifloxacin	Aerobic Gram-positive, Gram-negative species, some anaerobic Gram-negative species	Topoisomerase II (DNA gyrase), topoisomerase IV	DNA replication, SOS response, cell division, ATP generation, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope andredox-responsive two-component systems
Trimethoprim–sulfamethoxazole	DNA synthesis inhibitor	Co-trimoxazole	Aerobic Gram-positive and Gram-negative species	Tetrahydrofolic acid synthesis inhibitors	Nucleotide biosynthesis and DNA replication
Aminoglycosides	Protein synthesis inhibitors	Gentamicin, tobramycin, streptomycin and kanamycin	Aerobic Gram-positive and Gram-negative species	30S ribosome	Protein translation (mistranslation by tRNA mismatching), SOS response, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Tetracyclines	Protein synthesis inhibitors	Tetracycline and doxycycline	Aerobic Gram positive and Gram-negative species	30S ribosome	Protein translation (through inhibition of amino acyl tRNA binding to ribosome)
Macrolides	Protein synthesis inhibitors	Erythromycin and azythromycin	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of elongation and translocation steps) and free tRNA depletion
Streptogramins	Protein synthesis inhibitors	Pristinamycin, dalfopristin and quinupristin	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of initiation, elongation and translocation steps) and free tRNA depletion
Phenicols	Protein synthesis inhibitor	Chloramphenicol	Some Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of elongation step)
β-lactams	Cell wall synthesis inhibitors	Penicillins (penicillin, ampicillin, oxacillin), cephalosporins (cefazolin, cefoxitin ceftriaxone, cefepime) and carbapenems (imipenem)	Aerobic and anaerobic Gram-positive and Gram-negative species	Penicillin-binding proteins	Cell wall synthesis, cell division, autolysin activity (regulated by LytSR–VncRS two-component system), SOS response, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two component systems
Glycopeptides	Cell wall synthesis inhibitor	Vancomycin; teicoplanin	Gram-positive species	Peptidoglycan units (terminald-Ala-d Ala dipeptide)	Cell wall synthesis, transglycosylation, transpeptidation and autolysin activation (VncRS two-component system)

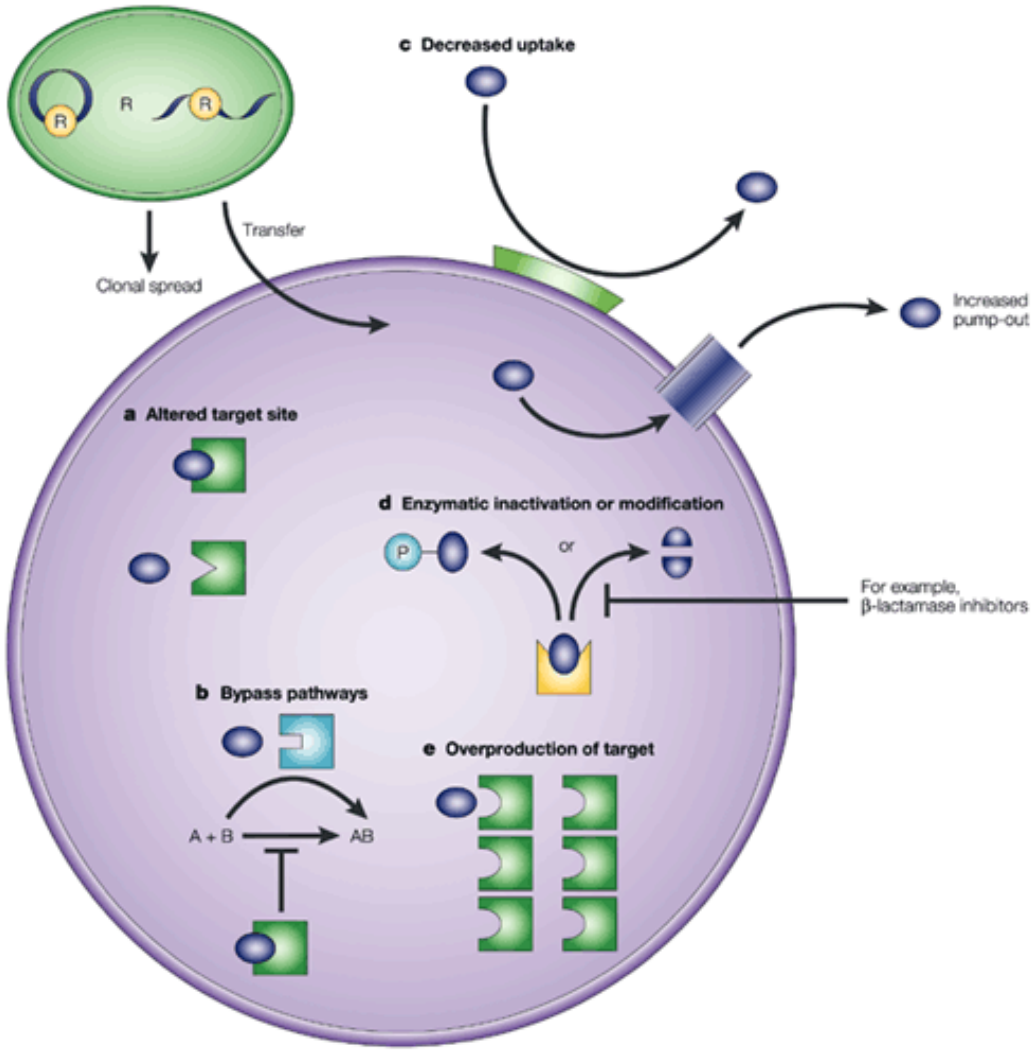

Figure I.2.3 | Bacterial mechanisms of antimicrobial resistance

a) The site of action (enzyme, ribosome or cell-wall precursor) can be altered. For example, acquiring a plasmid or transposon that codes for a resistant dihydrofolate reductase confers trimethoprim resistance to bacteria 52. b) The inhibited steps can be by-passed. c) Bacteria can reduce the intracellular concentration of the antimicrobial agent, either by reducing membrane permeability. d) They can inactivate the drug. e) The target enzyme can be overproduced by the bacteria. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery] (The future challenges facing the development of new antimicrobial drugs, 1: 895-910), copyright (2002) (Coates et al., 2002)

Table I.2.2 | Biochemical mechanisms of antimicrobial resistance

Antibiotic Class	Resistance Type	Resistance Mechanism	Common Example (s)
Aminoglycosides	Decreased uptake	Changes in outer membrane permeability	*P. aeruginosa*
		Phosphotransferase	Wide range of enteric negative bacteria
	Enzymatic modification (AMEs)	Adenyltransferase	Wide range of enteric negative bacteria
		Acetyltransferase	Wide range of enteric negative bacteria
		Bifunctional enzyme	*S. aureus*, *E. faecium* and *E. faecalis* *aac*(6′)-*aph*(2′′)
β-lactams	Altered PBPs	PBP2a (additional PBP)	*mec*A in *S. aureus* and coagulase-negative staphylococci
		PBP2x, PBP2b, PBP1a	*S. pneumoniae*
		PBP5 (point mutation)	*E. faecium*
	Enzymatic degradation (β-lactamases)	Ambler class A	TEM-1 in *E. coli, H. influenzae* and *N. gonorrhoe*; SHV-1 in *K. pneumoniae*; K-1 (OXY-1) in *K. oxytoca*; Extended-spectrum β-lactamases (TEM–3+, SHV–2+, and CTX-M types) *K. pneumoniae* and *E. coli*
		Ambler class B	Ccr-A in *B. fragilis*
		Ambler class C	AmpC in *E. cloacae*, *C. freundii, S. marcescens, M. morganii*
		Ambler class D	OXA-1 in *E. coli*
Chloramphenicol	Enzymatic degradation	CAT	CAT in *S. pneumonia*
	Efflux	New membrane transporters	*cml*A and *flo*-encoded efflux in *E. coli* and *Salmonella* spp.
Glycopeptides	Altered target	Altered peptidoglycan cross-link target (D-Ala-D-Ala to D-Ala-DLac or D-Ala-D-Ser) encoded by complex gene cluster	*van*A and *van*B gene clusters in *E. faecium* and *E. faecalis*
	Target overproduction	Excess of peptidoglycan	Glycopeptide “intermediate” strains of *S. aureus*
Macrolides-lincosamides-streptogramins B	Altered target	Methylation of ribosomal active site with reduced binding	*erm*-encoded methylases in *S. aureus*, *S. pneumoniae*, and *S. pyogenes*
Macrolides	Efflux	Mef type pump	mef-encoded efflux in *S. pneumoniae*and *S. pyogenes*
Streptogramins Streptogramin A	Enzymatic degradation	Acetyltransferase	*vat*A, *vat*B, and *vat*C in *S. aureus* *E. faecium vat*D and *vat*E
Quinolones	Altered target	Mutation leading to reduced binding to active site(s)	Mutations in *gyr*A in enteric Gram-negative bacteria and *S. aureus* Mutations in *gyr*A and *par*C in *S. pneumoniae*
	Efflux	New membrane transporters	*Nor*A in *S. aureus*

Antibiotic Class	Resistance Type	Resistance Mechanism	Common Example(s)
Tetracyclines	Efflux	New membrane transporters	*tet* genes encoding efflux proteins in Gram-positive and Gram-negative bacteria
	Altered target	Production of proteins that bind to the ribosome and alter the conformation of the active site	*tet*(M) and *tet*(O) in diverse Gram-positive and Gram-negative bacteria species
Sulfonamides	Altered target	Mutation or recombination of genes encoding DHPS; Acquisition of new low-affinity; DHPS genes	*S. aureus*, *S. pneumoniae*, *H. influenzae*
Trimethoprim	Altered target	Mutations in gene encoding DHFR	*sul*1 and *sul*2 in enteric Gram-negative bacteria
		Acquisition of new low-affinity; DHFR genes	*S. aureus, S. pneumoniae, H. influenzae*
	Overproduction of target	Promoter mutation leading to overproduction of DHFR	*dhfr*1 and *dhfr*2 encoded, found in a wide range of species *E. coli*

I.2.4.1. Intrinsic resistance

Intrinsic resistance is the innate ability of a bacterial species to resist activity of a particular antimicrobial agent by naturally occurring genes found on the host's chromosome, such as, AmpC β-lactamase of Gram-negative bacteria and many MDR (Multiple Drug Resistance) efflux systems (Lupo et al., 2012). Further example of intrinsic resistance is the natural resistance of anaerobes to aminoglycosides and Gram-negative bacteria against vancomycin. Some species of bacteria are innately resistant to ≥ 1 class of antimicrobial agents. In these cases, all strains of that bacterial species are similarly resistant to all the members of those antibacterial classes (Tenover, 2006).

It can also be called "insensitivity" since it occurs in organisms that have never been susceptible to that specific antimicrobial agent. Such natural insensitivity can be due to: (a) absence of affinity of the drug for the bacterial target, (b) inaccessibility of the drug into the bacterial cell, (c) extrusion of the drug by chromosomally encoded active exporters, (d) innate production of enzymes that inactivate the drug (Giguère et al., 2006).

The intrinsic resistance understanding of pathogen bacteria is important in order to avoid inappropriate and ineffective treatments. For bacterial pathogens, which are naturally resistant to a large number of classes of antimicrobials, such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* this situation can pose a limitation in the range of options for treatment and thus consequently further increase the risk for occurrence of acquired resistance (Fajardo et al., 2008).

I.2.4.2. Acquired resistance

Of greater concern are cases of acquired resistance, where originally bacteria are susceptible but become resistant to an antimicrobial agent, proliferate and spread under the selective pressure of use of that agent (Tenover, 2006).

Acquired antimicrobial resistance can be due to different mechanisms. Firstly, drug inactivation, such as by β-lactamases and aminoglycoside-modifying enzymes, which confer resistance to β-lactams and aminoglycosides, respectively; secondly, drug target modification by mutation, for example RNA polymerase or DNA topoisomerase modifications, conferring resistance to rifampicin and quinolones, respectively; thirdly, drug target protection, as ribosomal methylation conferring resistance to aminoglycosides or macrolides, or topoisomerase protection by Qnr proteins conferring resistance to quinolones; fourthly, drug target bypass, such as peptidoglycan synthesis by a novel penicillin-binding-protein, PBP2a, conferring resistance to β-lactams; fifthly, impermeability, for example by loss of outer membrane porin channels, conferring resistance to carbapenems; and finally drug efflux, for example by Tet major facilitator superfamily (MFS)-type pumps, conferring resistance to tetracyclines, or by tripartite resistance-nodulation-cell division superfamily (RND)-type pumps, conferring a multidrug resistance phenotype. This variety of resistance mechanisms reveals the broad repertoire of available agents, the multiplicity of their mechanisms of action, the biological diversity of bacteria, and the plasticity of their genomes (Rossolini & Thaller, 2010). Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacteria with multidrug resistance (defined as resistance to ≥ three antibacterial drug classes) have become a cause for alarming concern, principally in hospitals and other healthcare institutions where they tend to happen most frequently (Tenover, 2006). Resistance to antimicrobial agents could be due to an innate property of the bacterium, or a consequence of mutation or horizontal gene transfer (Coates et al., 2002).

Mutation. Susceptible bacteria can acquire resistance to an antimicrobial agent via new spontaneous mutations (McManus, 1997). Such mutations may cause resistance by (a) changing the target protein to which the antimicrobial agent binds by altering or eliminating the binding site (e.g., modify in penicillin-binding protein 2b in pneumococci, which results in penicillin resistance), (b) upregulating the production of enzymes that inactivate the antimicrobial agent (e.g., erythromycin ribosomal methylase in staphylococci), (c) downregulating or changing an outer membrane protein channel that the drug requires for cell entry (e.g., OmpF in *E. coli*), or (d) upregulating pumps that eject the drug from the cell (efflux of fluoroquinolones in *S. aureus*) (McManus, 1997). In each of these circumstances, bacterial strains carrying resistance-conferring mutations are selected by antimicrobial use, which kills the susceptible strains but permits the newly resistant strains to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is designated vertical evolution (Tenover, 2006).

Horizontal gene transfer. Bacteria can also gain resistance genes through the acquisition of new genetic material from other resistant organisms. This is called horizontal evolution, and may happen between strains of the same species or also between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and transformation (Figure I.2.4) (McManus, 1997). For all these processes, transposons may facilitate the incorporation and transfer of the acquired resistance genes into the host's genome or into plasmids. During transduction, resistance genes are transferred from one bacterium to another via bacteriophage (bacterial viruses). Researchers supposed that this process is now relatively rare event. During conjugation, a Gram-negative bacterium transfers plasmid-containing resistance genes to an adjacent bacterium; frequently via an elongated proteinaceous structure known as a pilus, which connects the two organisms. Commonly, conjugation among Gram-positive bacteria is initiated by production of sex pheromones by the mating pair, which facilitate the clumping of donor and recipient organisms, permitting the exchange of DNA. Lastly, transformation, i.e., the mechanism whereby bacteria acquire and incorporate DNA segments from other bacteria that have released their DNA complement into the environment after cell lysis, can move resistance genes into previously susceptible strains (Tenover, 2006).

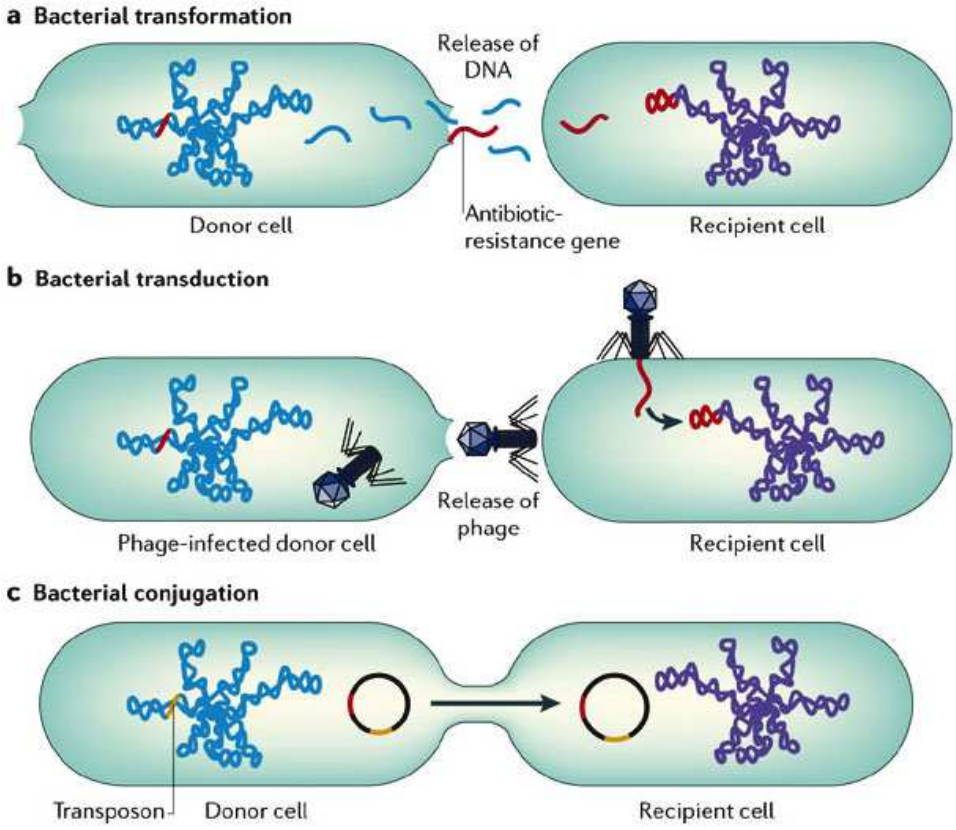

Figure I.2.4 | Horizontal gene transfer between bacteria

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I.2.5. Resistance to β-lactam antimicrobials

The insertion of β-lactam antimicrobials into the healthcare system in the last periods of World War II represents one of the greatest contributions to medical science in recent history. In fact, β-lactam antimicrobials are an important part of our antimicrobial armamentarium of the infectious disease specialist, used to treat a variety of Gram-negative and Gram-positive infections (Bosso, 2005).

Nowadays, β-lactams remain the most broadly consumed antimicrobials owing to their comparatively high effectiveness, low cost, ease of delivery and minimal side effects (Wilke et al., 2005). The low cost of production of β-lactam antimicrobials allows for a wide availability; thus, it is imperative that we preserve the power of this valuable clinical resource (Wilke et al., 2005).

These antimicrobial agents represent >65% of the world antimicrobial market with >50 marketed drugs of this class including the penicillins, cephalosporins, carbapenems, monobactams (Poole, 2004) and more lately the penicillin-cephalosporin hybrids, the penems (e.g.impenem) (Figure 1.2.5) (Dalhoff & Thomson, 2003).

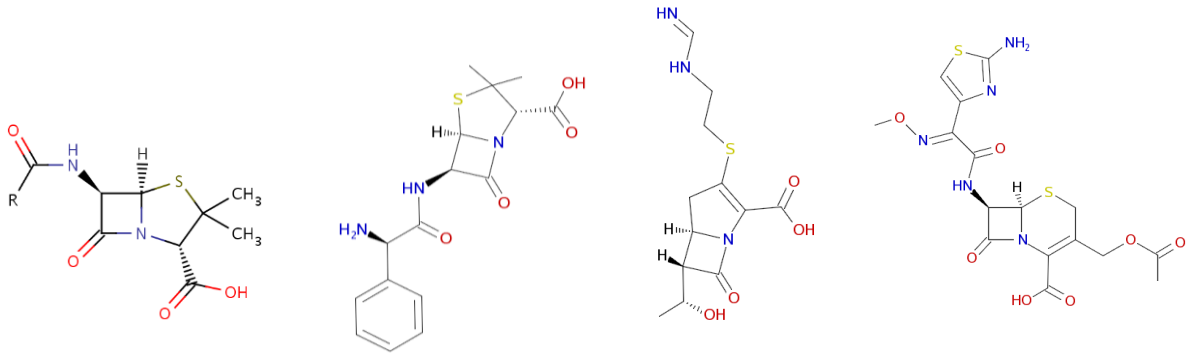

Figure I.2.5 | Chemical structure of β-lactam antimicrobials
From the left to the right: penicillin, ampicillin, imipenem and cefotaxime

Described by a four-membered β-lactam ring, these agents target the bacterial enzymes of cell wall biosynthesis (the penicillin-binding proteins, PBPs) (Essack, 2001; Siu, 2002). Commonly, resistance to β-lactam antimicrobials is observed in Gram-negative and Gram-positive (Poole, 2004) bacterial pathogens and performs as a result of drug inactivation by β-lactamases, target site (i.e. PBP) alterations, diminished permeability and efflux (Siu, 2002).

First occurrence in the early 1980s following the widespread use of broad-spectrum β-lactams, Extended-Spectrum-Beta-Lactamases (ESBLs) represent an important problem in the use of β-lactams to treat infectious diseases given their broad substrate specificity and ability to hydrolyze many of the extended-spectrum, third-generation cephalosporins (Poole, 2004). In addition to ESBLs, other beta-lactamases of great interest are AmpC β-lactamases and carbapenemases (Poole, 2004).

ESBLs are plasmid-encoded enzymes in the *Enterobacteriaceae,* being frequently found in *Escherichia coli.* ESBLs confer resistance to a variety of β-lactams, including penicillins, 2nd-, 3rd- and 4th-generation cephalosporins and monobactams. ESBL-producing organisms are frequently multiresistant, exhibiting resistance to other antimicrobial classes (fluoroquinolones, aminoglycosides and trimethoprim-sulphamethoxazole) due to associated resistance mechanisms, which may be either chromosomally- or plasmid-encoded (Paterson & Bonomo, 2005).

In the *Enterobacteriaceae,* the most commonly encountered ESBLs belong to the TEM, SHV and CTX-M families (Paterson & Bonomo, 2005). The SHV and TEM ESBL types were the main ESBL variants found in *E. coli* causing nosocomial infections; they emerged by mutations of the broad spectrum TEM-1 and SHV-1 genes that were then transferred between bacteria through plasmids, which were in turn spread by clonal distribution among hospitals and countries. However, in the last decade, the ESBL epidemiology in humans was modified and successful international bacterial clones harbouring members of the CTX-M family have emerged and spread globally. The CTX-M genes are also located on highly transmissible plasmids, thus facilitating fast and efficient spread of resistance. Based on their amino-acidic sequence diversity, the vast number (more than 50) of CTX-M variants described so far have been classified into 5 major groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25. The CTX-M-β-lactamases have become the most prevalent ESBLs in human *Enterobacteriaceae* worldwide (Pallecchi et al., 2007). Figure I.2.6 showed the distribution of ESBL-producing *E. coli* resistant to 3rd generation cephalosporins in Europe in 2011 (EARS-Net results, 2011).

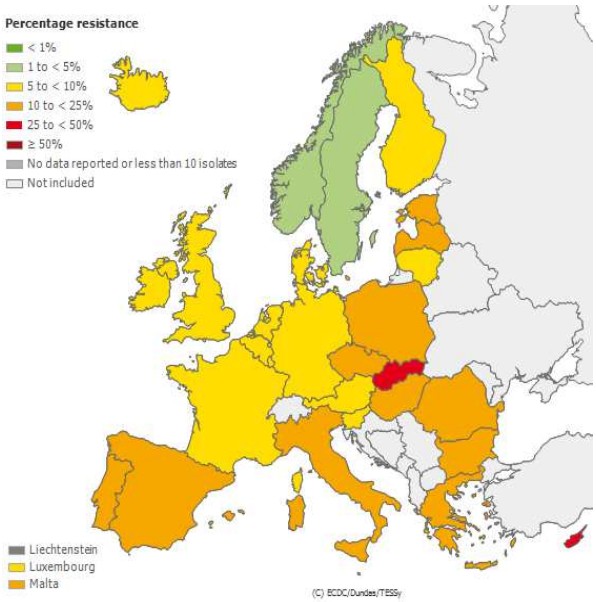

Figure I.2.6 | Distribution of ESBL-producing *E. coli* resistant to 3rd generation cephalosporins in Europe in 2011

I.2.6. Resistance to glycopeptide antimicrobials

During a long time, glycopeptide antimicrobials have stood as an exception to the resistance phenomenon. The lack of important levels of clinical resistance to the two most frequently used members, vancomycin and teicoplanin, led to their adoption as drugs of last resort for the treatment of otherwise resistant and deadly infections (James et al., 2012). However, recent years have finally brought the emergence of resistance to these significant drugs. These agents are the most important

class of drugs used in the treatment of resistant bacterial infections. After more than a half century of clinical use, the occurrence of glycopeptide-resistant Gram-positive pathogens such as vancomycin-resistant enterococci (VRE) and vancomycin-resistant *Staphylococcus aureus* (VRSA) continues to be a serious global challenge to public health at a time when few new antimicrobials are being developed (James et al., 2012).

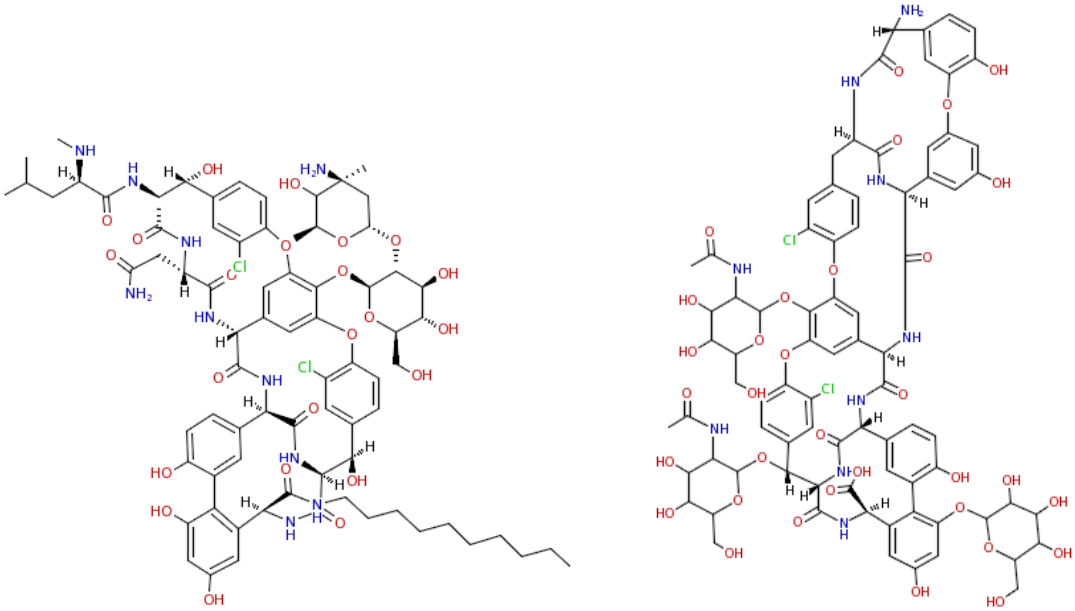

Figure I.2.7 | Chemical structure of glycopeptide antimicrobials
Left: vancomycin and right: teicoplanin

Concerning vancomycin, this agent is the leading member of the class of clinically significant glycopeptide antimicrobials (Figure I.2.7). Vancomycin was first revealed in 1956 (McCormick et al., 1955) and introduced into the clinic in 1958, though its structure was not established until nearly 30 years later in 1983 (Harris et al., 1983). In comparison to all other antimicrobial agents, the appearance of vancomycin resistance was long-delayed. Even after its first three decades of use, there was no notable resistance to vancomycin reported, and some even speculated that the development of resistance might be impossible (James et al., 2012). Resistance to glycopeptides was not reported for approximately 30 years after the introduction of vancomycin into clinical practice, due partly to limited use of the antimicrobial until the mid-1970s. The lengthy period was also due to the difficulties experienced by bacteria in developing mechanisms of resistance to an antimicrobial, which binds to an essential substrate in a biosynthetic pathway rather than to a protein or nucleic acid. Following publication in 1988 of reports of the first vancomycin-resistant enterococci (Leclercq et al., 1988), it was predicted that resistance might arise by one of four possible routes, including inactivation of the antimicrobial, sequestration of the antimicrobial in the outer cell wall layers by specific and nonspecific binding (the probable mechanism by which low-level resistance is achieved in staphylococci), by an increased production of those intermediates

to which vancomycin binds, or by a change in the target site (Reynolds & Courvalin, 2005). It was later recognized that the last mechanism would involve not only a new pathway to achieve a change of target but also elimination of at least part of the normal susceptible pathway (Reynolds et al., 1994a).

The appearance and spread of VRE are two very different stories often confused as one. The first and most well known tale emerged in the 1990s and involves the frequent isolation of VRE from healthy humans and animals in Europe (Van der Auwera et al., 1996). The emergence and spread of these strains was clearly tied to the use of the glycopeptide antimicrobial avoparcin in food animals (Wegener et al., 1999). The acknowledgement of this association finally led to the ban of avoparcin use in the European community. Curiously, true infections owing to VRE were rare in European hospitals during this time of widespread gastrointestinal colonization (Witte & Klare, 1995).

Figure I.2.8 show the distribution of vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* isolates in Europe 2011 (EARS-Net results, 2011)

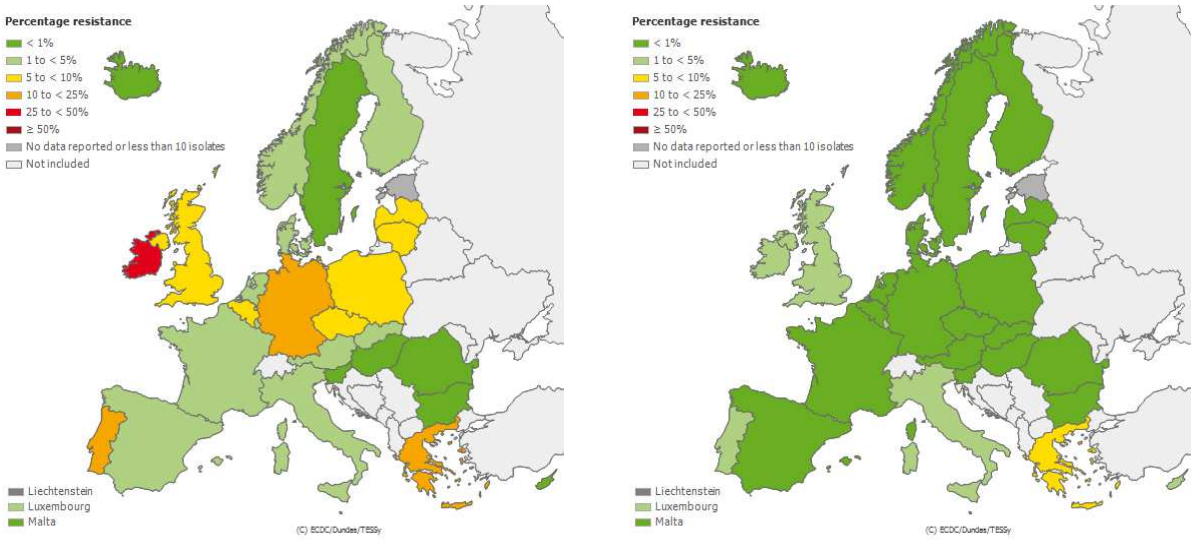

Figure I.2.8 | Distribution of vancomycin-resistant enterococci in Europe in 2011
Left *E. faecium* isolates and right *E. faecalis*

Contrarily, VRE occurred as a major problem in hospitalized patients in the United States of America (USA), where avoparcin had never been used (Rice, 2009). The USA strains were far more possible than European strains to be resistant to high concentrations of ampicillin (Descheemaeker et al., 1999; Sahm et al., 1999), which likely describes the frequent clinical association between VRE gastrointestinal colonization and clinical infection in the USA with the use of cephalosporins (Rice, 2001).

The activity of vancomycin is not achieved by the affinity for a target enzyme but by the substrate specificity of the enzymes that determine the structure of peptidoglycan precursors because

this agent does not interact with cell wall biosynthetic enzymes but forms complexes with peptidoglycan precursors (Courvalin, 2006).

Resistance to vancomycin can be explained by the presence of operons that encode enzymes (i) for synthesis of low-affinity precursors, in which the C-terminal d-Ala residue is substituted by d-lactate (d-Lac) or d-serine (d-Ser), therefore altering the vancomycin-binding target; and (ii) for elimination of the high-affinity precursors that are naturally produced by the host, thus removing the vancomycin-binding target (Arthur et al., 1996b).

High levels of resistance to vancomycin and teicoplanin in *E. faecium* and *E. faecalis* is characteristic of the VanA type, in which the acyl-D-Ala-D-Ala terminus of peptidoglycan precursors to which glycopeptides bind has been substituted by acyl-D-Ala-D-lactate with the loss of a crucial hydrogen bond in the binding site. This results in a more-than-1,000-fold lowering of the affinity of vancomycin for its target. The substitution is achieved by the activity of two enzymes, a D-lactate dehydrogenase and a ligase with specificity directed towards synthesis of D-Ala-D-lactate (D-Lac) rather than D-Ala-D-Ala (Bugg et al., 1991). An important aspect of the resistance mechanism is the elimination of D-Ala-D-Ala by a DD-dipeptidase and/or of precursors containing this moiety by a DD-carboxypeptidase that eliminates the C-terminal D-Ala (Arthur et al., 1994; Reynolds et al., 1994a). This mechanism has been significantly reviewed (Arias & Murray, 2012; Arthur & Courvalin, 1993; Arthur et al., 1996b; Gholizadeh & Courvalin, 2000; Reynolds & Courvalin, 2005; Woodford, 2001).

The other mechanism, which confers a low level of resistance to vancomycin and susceptibility to teicoplanin, involves replacement of D-Ala-D-Ala by a different dipeptide, D-Ala-D-Ser, rather than by the depsipeptide D-Ala-D-Lac (Reynolds et al., 1994b). This mechanism is used by the intrinsically glycopeptide-resistant *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* (VanC type) and is present in the VanE and VanG types in which *E. faecalis* has possessed the genes encoding vancomycin resistance. Some of the enzymes involved in this resistance mechanism are different from those involved in high-level resistance, showing a second route by which resistance has evolved. The basis of resistance results from the six fold-lower affinity of vancomycin for acyl-D-Ala-D-Ser than for acyl-D-Ala-D-Ala because of the increased bulk of the hydroxyl-methyl group of serine relative to the methyl group of alanine (Reynolds & Courvalin, 2005).

The simultaneous production of precursors ending in d-Ala or d-Lac does not implicate to resistance (Arthur et al., 1996a). Under these circumstances, binding of glycopeptides to precursors that carry d-Ala-d-Ala inhibits peptidoglycan synthesis. The interaction of vancomycin with its target is prevented by the removal of the susceptible precursors that end in d-Ala (Reynolds et al., 1994b). Two enzymes are involved in this mechanism: the VanX D,D-dipeptidase, which hydrolyzes the d-Ala-d-Ala dipeptide synthesized by the host d-Ala:d-Ala ligase (Ddl) (Reynolds et al., 1994a), and the VanY D,D-carboxypeptidase, which removes the C-terminal d-Ala residue of late peptidoglycan precursors when elimination of d-Ala-d-Ala by VanX is incomplete (Arthur et al.,

1998). As opposed to VanA-type resistance, in which the VanX and VanY activities are catalyzed by 2 enzymes (Arthur et al., 1998), VanXYC has both D,D-dipeptidase and D,D-carboxypeptidase activity (Courvalin, 2006; Reynolds et al., 1999).

I.2.7. Virulence and pathogenicity

I.2.7.1. Expression of microbial virulence determinants

Bacterial virulence factors permit a host to replicate and disseminate within a host in part by subverting or eluding host defenses. The capability of pathogenic bacteria to invade susceptible host and cause disease is determined by multiple virulence factors acting individually or together at different stages of infection (Wu et al., 2008).

Frequently, virulence factors are involved in concealing the bacterial surface from the host's defense mechanisms or in direct interactions with the host tissues (Wu et al., 2008). Bacterial virulence factors can be roughly divided into several groups on the basis of the function and mechanism of virulence (Finlay & Falkow, 1997): (a) Membrane proteins, which play roles in adhesion, colonization, and invasions, promote adherence to host cell surfaces, promote intercellular communication and are responsible for resistance to antimicrobials; (b) polysaccharide capsules that surround the bacterial cell and have antiphagocytic properties; (c) secretory proteins, for example toxin, which can alter the host cell environment and are responsible for some host cell–bacteria interactions. Bacterial pathogens use different secretion systems, most usually types I–IV (Tomich et al., 2007), to transport protein toxins from their cytoplasm into extracellular matrix or the host (China & Goffaux, 1999). Autotransporters (ATs) are virulence proteins transported by a diversity of pathogenic Gram-negative bacteria across the cell envelope to the cell surface or extracellular environment. ATs include a family of proteins collectively secreted by the type V pathway (van Ulsen et al., 2006); (d) cell wall and outer membrane components, such as lipopolysaccharide (LPS or endotoxin) and lipoteichoic acids. Naturally, Gram-positive bacteria are bounded by a thick cell wall that has a low permeability to the surrounding environment, whereas in Gram-negative bacteria the main outer membrane glycolipid, LPS, can defend against complement-mediated lysis. LPS activates the host complement pathway and is a powerful inducer of inflammation (Finlay & Falkow, 1997); (e) additional virulence factors, for example biofilm forming proteins and siderophores. Biofilm structure confers a selective advantage for persistence under environmental conditions and for resistance to antimicrobial agents and also facilitates colonization in the host by the bacteria. Furthermore, some bacterial virulence factors act as simulators of mammalian proteins to destabilize normal host cell processes. The virulence factor concept has been a powerful engine in driving research and the intellectual flow in the fields of microbial pathogenesis and infectious diseases (Wu et al., 2008).

I.2.7.2. Bacterial Pathogenesis

Pathogenicity represents another bacterial lifestyle, with the host serving simply as an additional ecological niche. For example, the evolutionary forces driving the adaptation of microorganisms to environmental niches function the same way in the evolution of pathogens. Therefore, as for all microbial genomes, bacterial pathogens have progressed by three main mechanisms: (i) modification of existing genes, (ii) loss of genes no longer under selection, and (iii) gain of genes that confer benefit in their current ecological niche (Gal-Mor & Finlay, 2006).

In 1990, Hacker and co-workers defined the term pathogenicity islands (PAI), describing two large unstable regions on the chromosome of uropathogenic *E. coli* (UPEC) (Hacker et al., 1990). Nowadays, this term is usually used to describe regions in the genomes of certain pathogens that are normally absent from non-pathogenic strains of the same or closely related species and that carry large continuous blocks of virulence genes. PAIs are considered to be a subclass of genomic islands that are acquired by horizontal gene transfer via transduction, conjugation and transformation, and provide 'quantum leaps' in microbial evolution. Data based on numerous sequenced bacterial genomes demonstrate that PAIs are present in a wide range of both Gram-positive and Gram-negative bacterial pathogens of humans, animals and plants. Recently, PAIs characterized in a expansive range of bacterial pathogens have not only led to the identification of many virulence factors used by these species, but also transformed our way of thinking about the evolution of bacterial pathogenicity (Gal-Mor & Finlay, 2006).

Despite the advances in prevention and treatment, bacterial pathogens still represent a major threat on human health worldwide. Researching virulence factors of pathogenic bacteria is crucial to get a better knowledge of pathogenesis and for identification of targets for novel drugs and design of new vaccines. Comparative genomics, transcriptomics, and proteomics have become the common tools in discovering the virulence factors in bacterial pathogens. Combination of these approaches will accelerate the developments of therapeutic drugs and vaccines in combating bacterial diseases (Wu et al., 2008).

I.2.8. Genomic approaches as a tool against resistance

The increase in antimicrobial drug resistance, together with the failure of conventional research efforts to discover new antimicrobials, may eventually lead to a public health emergency that can drastically limit the ability to combat infectious disease. In this context, it is imperative to consider how to keep our ability to deal efficiently with bacterial infectious diseases (Hughes, 2003).

Red biotechnology is concerned with the discovery and development of innovative drugs and treatments. Advances in this and associated scientific fields have revolutionized the practice of medicine: newer and simpler tests for the more accurate diagnosis of disease; genetic and proteomic

analyses that allow disease prevention; more efficient methods for designing and making drugs that are targeted at the molecular level and therefore conceivably more effective but less toxic; the possibility of gene therapy to cure diseases that are previously incurable. Though genomics and its applications usually come to mind when medical biotechnology is mentioned, it should be remembered that other disciplines such as bioinformatics, nanotechnology, fermentation technology and cell technology also play a significant role in the development of the field (Hsu et al., 2007).

In fact, the increasing resistance of pathogenic bacterial to usual antimicrobials has created need for new antimicrobial agents. Computational approaches such as bioinformatics is participating and accelerating the antimicrobial drug discovery process (Hammami & Fliss, 2010). Databases and bioinformatics tools that contain genomic, proteomic and functional information have become crucial in antimicrobial drug studies. Several common databases are used in this field, such as UniProt (Wu et al., 2006) and PDB (Dutta et al., 2007). During the past decade, databases dedicated to antimicrobial peptides (AMPs) have been developed and a total of seven antimicrobial databases are currently described in the Nucleic Acids Research Molecular Biology Database Collection (http://www3.oup.co.uk/nar/database/a/) (Zasloff, 2002).

Pursuing for similarities between biological sequences is the major way by which bioinformatics approach contributes to our understanding of biology. Of the various informatics' tools developed to achieve this task, the most extensively used is the basic local alignment search tool, also as known as BLAST (Pertsemlidis & Fondon, 2001).

Loading all of the sequence and other associated information into a relational database allows the integration of various tools including homology search, multiple sequence alignment, phylogenies, physicochemical profiles, etc. Homology search engines such as BLAST (Altschul et al., 1997) and FASTA (Pearson & Lipman, 1988) need the building of organized database.

These databases can be created for individual species genomes and proteomes, and also the entire combined collections of DNA sequences and proteins. Such resources were used in some reports to determine the type of the β-lactam genes or also integrons and gene cassettes (Gonçalves et al., 2012; Radhouani et al., 2012). Furthermore, sequences can also be exported into programs for multiple sequence alignment and phylogenetic analyses such as CLUSTALW (Larkin et al., 2007) and PHYLIP (Felsenstein, 1989), respectively. For example, in antimicrobial resistance studies CLUSTALW2 is usually used to determine mutations in *gyr*A and *par*C that confer resistance to fluoroquinolones; and also *amp*C that confers resistance to broad-spectrum cephalosporins (Gonçalves et al., 2012; Radhouani et al., 2012).

Besides, the primary sequence of peptides can be investigated using tools such as ProtParam (Gasteiger et al., 2005) (http://www.expasy.org/tools/protparam.html) and pepstats (Rice et al., 2000) (EMBOSS package: http://www.ebi.ac.uk/ emboss/). Secondary structure can be predicted using programs such as NNPREDICT (Kneller et al., 1990) (http://www.cmpharm.ucsf.edu/~nomi/ nnpredict.html) and SCRATCH servers (Cheng et al., 2005) (http://scratch.proteomics.ics.uci.edu/)

Approach in genomics is requiring a catalogue of potential new targets for antimicrobial and vaccine therapy. Performances in molecular genetics are facilitating the rapid evaluation of the essentiality of these targets on a genomic scale. Comparative genomics and molecular genetics are being used to create lists of essential new targets for compound screening programmes. Combinatorial chemistry and structural biology are being applied in order to rapidly explore and optimize the interactions between lead compounds and their biological targets. Numerous compounds, which have been identified from target-based screens, are currently in development, but technical and economic constraints might result in a trickle, rather than a flood, of new antimicrobials onto the market in the near future (Hughes, 2003).

I.2.8.1. Genomics in surveillance and control of resistance

For the surveillance and control of antimicrobial resistance, which can no longer count on the simple phenotypic characterization of bacterial isolates, discovering the epidemiology of resistant strains and resistance genes is paramount importance. Molecular epidemiology is the research that investigates the epidemiology of resistant strains and resistance genes by describing them at the molecular level, and it has delivered major breakthroughs in the understanding of this phenomenon, with practical implications for resistance control strategies (Hayden, 2000).

The knowledge provided by molecular analysis with genetic ones is being the most versatile and of highest resolution. From this point of view, full genomic researches of major resistant clones can be very helpful for understanding their evolution and lifestyle and would be the excellent standard for their comparison. Genomic research can also develop sets of molecular probes to identify more easily with precisely identification resistant clones of high spreading propensity and clinical impact, which is essential in infection control practices. These probes can be utilized by reference laboratories or even by the largest diagnostic laboratories, in multiplexed amplification or DNA microarray tools, for identification of such 'high risk' resistant clones involved in hospital or community outbreaks (Rossolini & Thaller, 2010).

Recently, a considerable effort has been undertaken to sequence the genomes of multiresistant strains of bacterial pathogens in order to identify high risk resistant clones and understanding their evolution (http://www.ncbi.nlm.nih.gov/bioproject), and also to sequence plasmids involved in the dissemination of important resistance determinants, such as ESBLs, AmpC-type β-lactamases or (quinolone resistance) Qnr proteins (Carattoli, 2009).

The most successful European antimicrobial resistance surveillance scheme is the European Antimicrobial Resistance Surveillance System/network (EARSS/EARSNet), which was established in 1998 and is now funded by the European Centre for Disease Prevention and Control ECDC. EARS-Net collects data on antimicrobial resistances in indicator bacteria exclusively from invasive (bloodstream) infections currently covering *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*/*E. faecium*, *Klebsiella pneumonia* and *Pseudomonas*

aeruginosa. Inter-country comparison of collected data in the given setting reveals some drawbacks and limitations (Werner, 2012).

I.2.8.2. Genomics in understanding of resistance mechanisms

In the process of discovery and development of antimicrobial drugs, understanding resistance mechanisms to novel drugs is imperative knowledge for the prediction of resistance evolution, antimicrobial policies and resistance surveillance and control strategies. Genome-scale research may offer relevant perceptions into unknown mechanisms of antimicrobial resistance (Rossolini & Thaller, 2010). Recently, a comparative genomic research between vancomycin resistant and susceptible *S. aureus* strains show the role of mutations in the genes encoding the two-component regulatory system that confers this resistance phenotype, which is related with clinical failures of glycopeptide therapy (Cui et al., 2009). Furthermore, a similar comparative genomic analysis, realized using array-based methodology, provided insights into mutational mechanisms that, in *S. aureus*, could be responsible for the emergence of reduced susceptibility to daptomycin (Friedman et al., 2006). Another genome-scale investigation advanced our knowledge of resistance mechanisms. Breidenstein and co-workers (Breidenstein et al., 2008) identified a number of new genes that, when inactivated, were associated with decreased susceptibility to fluoroquinolones in *P. aeruginosa*.

Transcriptomics at a genome-wide scale has also been used successfully to research resistance mechanisms. Transcription profiling following ciprofloxacin exposure has revealed a significant role of the *mfd* gene, which encodes a transcription-repair coupling factor involved in strand-specific DNA repair, in the development of fluoroquinolone resistance in *Campylobacter jejuni* (Han et al., 2008). Transcriptome approach of *S. aureus* has discovered a role for several genes, including the *vra*SRcell wall regulon, in the bacterial response to cationic antimicrobial peptides, as well as a role of the VraDE putative ATP-binding cassette (ABC) transporter in conferring resistance to bacitracin (Pietiainen et al., 2009)**.**

I.2.8.3. Genomic knowledge and antibacterial drug discovery

During recent years, knowledge on bacterial genomes has progressed at a fast pace, with almost 1,500 completed bacterial genomes and more than 600 additional genome projects in progress at the beginning of 2010 (http://www.ncbi.nlm.nih.gov/bioproject).

Nowadays, microbial genomics has evolved from the long drawnout individual genome sequencing projects in the past to a high level of technological advancement, where sequencing and comparing the genomes of numerous strains of a single pathogen is realized in a very short period of time (Okeke et al., 2005).

Actually, since its very beginning, the advent of bacterial genomics was not only regarded as a fascinating scientific tool, but also increased the raised great hopes for renewing the golden era of antimicrobial discovery at a time when this is sorely needed because of the growing impact of bacterial resistance (Donadio et al., 2010).

The rationale behind this expectation was that comparative genomic analysis could reveal valuable information on bacterial genes that presumably encode proteins that are essential to survival or fitness of bacterial pathogens and do not have close eukaryotic counterparts; these proteins could then be potential targets for new antimicrobial agents, such as FtsZ protein which is a GTPase. Ftsz, and thus represent potential classes of new antimicrobials. Ftsz may also be a possible novel target for antituberculous agents (Reynolds et al., 2004). FabI enzyme is another protein that is a potential target for triclosan (antiseptic) and isoniazid (the anti-Mycotuberculosis agent) (Heath et al., 2000)

After the identification of the potential targets, many bottlenecks need to be overcome in the process of developing new drugs; especially, the necessary to set up high-throughput screening of banks of small molecules to acquire potential hits, which has prompted great efforts in the fields of functional and structural genomics. Finally, cloning and sequencing metagenomes from various ecological niches in which antimicrobial-producing bacteria are estimated to be present could expose new antimicrobial synthetic gene clusters from unknown or uncultured bacteria (Rossolini & Thaller, 2010).

Compared with genomics and transcriptomics, proteomics has the advantage of identifying proteins that are differentially expressed, not just merely transcriptional regulation. Moreover, it can define proteins that are differentially located or secreted to outside of the cell (the surfaceome). In several cases, genomics can predict the proteins that fall into these classes, however proteomics always shows some that are not predicted. Furthermore, only proteomics can define proteins that are post-translationally modified (Wu et al., 2008). Besides, the use of proteomics in the study of antimicrobial resistance in bacterial strains represents an important step to determine the metabolic pathways, which result in antimicrobial behavior. The identification of proteins related to the antimicrobial resistance represents valuable information on this problematic and the discovery of potential biomarkers related to the antimicrobial resistance is a fundamental resource in order to fight against this healthcare menace.

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I.2.10. References

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I.3. After genomics, what proteomics tools could help us understand the antimicrobial resistance of *Escherichia coli*?

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I.3.1. Abstract

Proteomic approaches have been considerably improved during the past decade and have been used to investigate the differences in protein expression profiles of cells grown under a broad spectrum of growth conditions and with different stress factors including antibiotics.
In Europe, the most significant disease threat remains the presence of microorganisms that have become resistant to antimicrobials and so it is important that different scientific tools are combined to achieve the largest amount of knowledge in this area of expertise. The emergence and spread of the antibiotic-resistant Gram-negative pathogens, such as *Escherichia coli*, can lead to serious problem public health in humans. *E. coli*, a very well described prokaryote, has served as a model organism for several biological and biotechnological studies increasingly so since the completion of the *E. coli* genome-sequencing project. The purpose of this review is to present an overview of the different proteomic approaches to antimicrobial-resistant *E. coli* that will be helpful to obtain a better knowledge of the antibiotic-resistant mechanisms. This can also aid to understand the molecular determinants involved with pathogenesis, which is essential for the development of effective strategies to combat infection and to reveal new therapeutic targets. This article is part of a Special Issue entitled: Proteomics: The clinical link.

I.3.2. Introduction

Since the first use of antibiotics in the 1940s, bacterial resistance progressively has become a common place amongst important human and animal pathogens. Antimicrobial-resistant microorganisms can acquire unexpected genetic background, consequently expressing new physiologic and molecular characteristics that could interfere with the organization of infectious diseases (Diniz et al., 2004; Linares-Rodriguez & Martinez-Menendez, 2005). Furthermore, the treatment with sub-inhibitory concentrations of certain antibiotics may also influence the virulence properties of microorganisms and their relationships with host immune defenses (Diniz et al., 2004; Lorian, 1993). The elucidation of the molecular details of drug resistance is helpful not only to further understand the antimicrobial-resistant mechanism(s), but also to lead the improvements in extending the efficacy of the current antimicrobials to the control of bacteria (Xu et al., 2006). Since the introduction of antibiotics in the last century, the occurrence of bacteria that present resistance to these compounds has rapidly outpaced the discovery and development of new antimicrobial agents (Levy & Marshall, 2004). The importance of understanding the bacterial physiology during the infection of the host is critical for the development of new antimicrobials or antibiotics that will reduce their burden upon the human health.

Extraintestinal pathogenic *E. coli* causes significant human and animal morbidity and mortality (Johnson et al., 2001). *Escherichia coli*, in humans, can bring a diversity of intestinal and extra-intestinal infections, for example diarrhea, meningitis, peritonitis, septicemia, urinary tract infection, and Gram-negative bacterial pneumonia. The enteric *E. coli* are classified on the basis of virulence characteristics into enterotoxigenic, enteropathogenic, enteroinvasive, verotoxigenic, enterohemorrhagic, and enteroaggregative *E. coli* (present only in humans). *E. coli* is a commensal inhabitant of the intestinal tracts of healthy animals that are used for food production, and so food of animal origin can be contaminated with *E. coli* during slaughter. Commonly, *E. coli* from meat has been linked with intestinal pathogenic *E. coli* (e.g., enteropathogenic, enterotoxigenic, and verotoxigenic *E. coli*), but recently, *E. coli* of animal origin has been shown to also be associated with extra-intestinal infections, such as urinary tract infections (Johnson et al., 2005). *E. coli* of wild animals, with or without antimicrobial resistance traits, represent a potential source of infections for humans and colonization (Bonnedahl et al., 2009). In fact, wild animals can be colonized with *E. coli* isolates, and may also act as an environmental reservoir and melting pot of bacterial resistance with a potential to re-infect human populations (Bonnedahl et al., 2009).

Mostly, the origin of *E. coli* that cause infection in humans can be attributed to many factors, mainly related to environmental contamination and its different ways of exposition/transmission, i.e. contaminated water ingestion, contaminated food, among others. However the importance of the animal reservoir of antimicrobial-resistant *E. coli* has not been quantified yet. Though, treatment alternatives for humans are compromised if the causative bacteria are previously multiple-

antimicrobial-resistant bacteria. To treat these infections, β-lactams are considerably used in human and veterinary medicine (Tracz et al., 2005). The production of extended-spectrum β-lactamases (ESBLs) by Enterobacteriaceae, in particular by *E. coli*, has been increasingly reported in the last few years in healthy animals (Ewers et al., 2011) and has caused a major concern in diverse countries, being frequently present in human infections (Paterson & Bonomo, 2005). Selective antimicrobial pressure, particularly caused by the intensive use of expanded-spectrum cephalosporins and cross-transmission, has been associated with the emergence and dissemination of ESBL-producing members of the family Enterobacteriaceae (Hsueh et al., 2005). Until the beginning of the 21st century, several nosocomial outbreaks of ESBL-producing organisms have been reported worldwide (Cantón et al., 2008). Incidence of ESBLs is high in nosocomial environments mainly because of the long-term exposure to antimicrobials and catheterization, among other factors (Bradford, 2001).

Qualitative and quantitative proteomic changes can be identified in a non-specific manner without making preconceived judgments about the potential significance of different components. At this time, proteomics is a challenging field that has been rising rapidly in the post-genomic era. Mass spectrometry performed in combination with several protein resolution methods and bioinformatics tools have become usual approaches to proteomic research (Jungblut et al., 2008). The array of protein species linked to antimicrobial resistance has been explored in a large diversity of microorganisms and with different antimicrobial agents (Andrade et al., 2008; Bore et al., 2007; Cash et al., 1999; Coldham & Woodward, 2004; Diniz et al., 2004; Lis & Bobek, 2008; McAtee et al., 2001; Pieper et al., 2006; Xu et al., 2006). Outer membrane proteins (OMPs) of Gram-negative bacteria are crucial molecules that interface the cell with the environment. Moreover, they are constantly assessing and responding to environmental modifications, thereby preserving the system regularly dealing with the existence of nutrients, minerals, and toxic compounds (Werner et al., 2009). The cells are involved in the adhesion to and in the invasion of host cells, in identifying the external physical and chemical conditions of the environment and in sending appropriate indicators to the cytoplasmic section, in rising defenses against the host responses and in toxicity. Therefore, surface proteins of Gram-negative bacteria such as *E. coli* have proteins that could be important targets for antimicrobials, drugs or detection systems. On the other hand, because surface proteins are likely to interact with the host immune system, they could become components of efficient vaccines (Rodriguez-Ortega et al., 2006). Furthermore, *E. coli* has been indicated as a standard organism by several structural and functional studies designed in order to obtain a better understanding of the biochemical and biophysical management of proteins in Gram-negative cell walls (Cirulli et al., 2007).

This review will consider the contribution of proteomic approaches to the study of antimicrobial-resistant *E. coli* in human isolates. The antimicrobial-resistant *E. coli* has been frequently used as a model organism in structural and functional studies aimed at understanding

bacterial physiology and gene expression. This is because *E. coli* not only has the entire genome sequence available, but is also one of the most important pathogenic bacteria, both for humans and animals. Also, since the evaluation of protein profiles in response to multiple stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance, it remains a significant and integrating approach to the development of new therapeutic strategies.

I.3.3. The Omics era

E. coli that belongs to the Enterobacteriaceae family has served as a model organism for several biotechnological studies. *E. coli* genome (Figure I.3.1) (Perna et al., 2001) analysis reveals the absence of introns and genes appear to be densely packed and overlapping with their neighbors, with an average gene measuring approximately 0.0004% of the mass of a typical *E. coli* cell (Schaechter & Grp, 2001). The complete *E. coli* genome sequence has been determined using two different strains of *E. coli* K-12, MG1655 and W3110. These strains diverged from a single ancestral strain about 50 years ago, resulting in slight but important differences like the large inversion involving the ribosomal RNA genes (Mori, 2004). *E. coli* genome varies between 4.6 and 5.6 Mb, indicating the presence of specific information of different amounts of strain which is considered as being 30% of the genome (Dobrindt et al., 2010). The existence of 4288 Open Reading Frames (ORFs) is also of high importance when it concerns the genomic diversity (Yan et al., 2002).

From an evolutionary perspective, *E. coli* has shared a common ancestor which is *Salmonella enterica* until ~ 150 million years ago. The divergence and the vertical transmission of existing genes are in the origin of mutational changes in bacterial populations, where the processes of recombination and rearrangement are included. On the other hand, the impact of the horizontal transfer has been slightly neglected due to the limitations found on the development of studies concerning this genetic behavior (Lawrence, 1999).

Comparative genome analysis revealed that the *E. coli* genome is constituted by the "core genome", the conserved part, and the flexible gene pool, interspersed regions of variable strain specific DNA. The second one comprises several foreign genes, including virulence gene acquired horizontally such as mobile elements like transposons and integrons. Recent studies on *E. coli* possessing an open pangenome, indicate that the species are evolving through processes of ordered gene acquisition as well as loss of genetic information and diversification. As a result, *E. coli* genome consists of clonally evolving DNA regions, which are disrupted upon the exchange of the present DNA segments through homologous insertion and recombination of horizontally acquired DNA. Furthermore, strain specific genomic regions are thought to be accumulating over time by repeated gene transfer processes (Dobrindt et al., 2010).

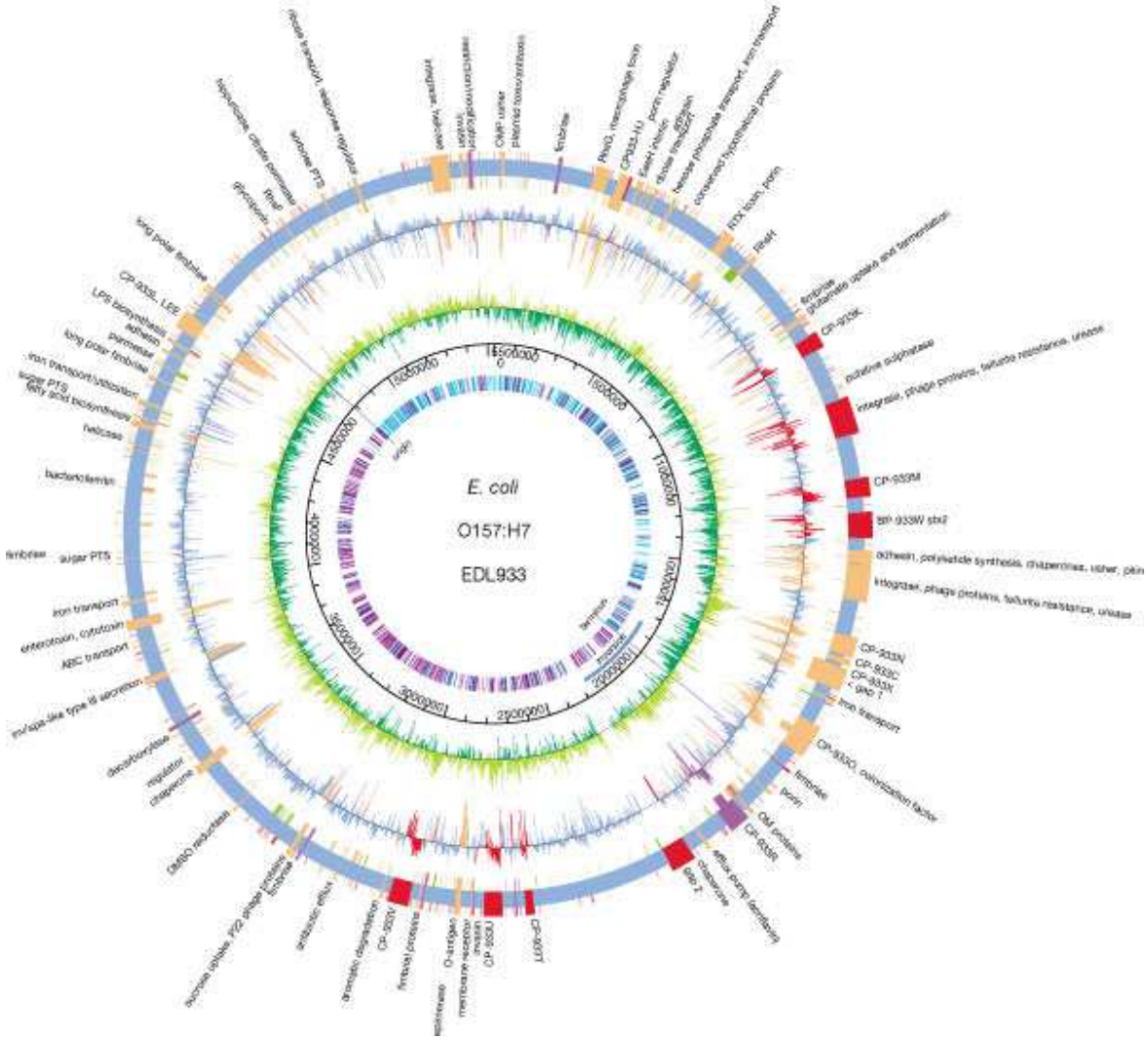

Figure I.3.1 | Circular genome map of *E. coli* O157:H7 EDL933 compared with MG1655

Outer circle shows the distribution of islands: shared co-linear backbone (blue); position of EDL933-specific sequences (O-islands) (red); MG1655-specific sequences (K-islands) (green); O-islands and K-islands at the same locations in the backbone (tan); hypervariable (purple). Second circle shows the G+C content calculated for each gene longer than 100 amino acids, plotted around the mean value for the whole genome, color-coded like outer circle. Third circle shows the GC skew for third-codon position, calculated for each gene longer than 100 amino acids: positive values, lime; negative values, dark green. Fourth circle gives the scale in base pairs. Fifth circle shows the distribution of the highly skewed octamer Chi (GCTGGTGG), where bright blue and purple indicate the two DNA strands. The origin and terminus of replication, the chromosomal inversion and the locations of the sequence gaps are indicated. Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Genome sequence of enterohemorrhagic *Escherichia coli* O157:H7, 409(25): 529-533), copyright (2001) (Perna et al., 2001)

E. coli is one of the best known prokaryotes that has been served as a model organism and therefore widely used in many experimental analysis, such as biochemical, biological, and biotechnological studies (Han & Lee, 2006). The full sequencing of the *E. coli* genome has facilitated the development of the proteomic analysis (Blattner et al., 1997; Pasquali et al., 1996; Tonella et al., 2001; Tonella et al., 1998) (Figure I.3.2). This organism has been characterized using the genome-wide scale taking into consideration its transcriptome, proteome, interactome, metabolome, and physiome by using the DNA microarray, two-dimensional gel electrophoresis

(2-DE) coupled with mass spectrometry (MS), liquid and gas chromatography coupled with MS, and bioinformatics (Butland et al., 2005; Lockhart & Winzeler, 2000; Nielsen et al., 1997; Pandey & Mann, 2000; Yu et al., 2004). The *E. coli* proteome was estimated to be constituted by 4285 proteins (Lopez-Campistrous et al., 2005) with pI between 3.38 and 13.0 and molecular mass between 1.59 and 248 kDa (Bjellqvist et al., 1994; Bjellqvist et al., 1993; Wilkins et al., 1998). The set of proteins found in the *E. coli* proteome can be divided into four subcellular compartments: cytosol (2885 known and predicted species), inner membrane (670 known and predicted species), outer membrane (87 known and predicted species) and periplasm (138 known and predicted species) (Lopez-Campistrous et al., 2005).

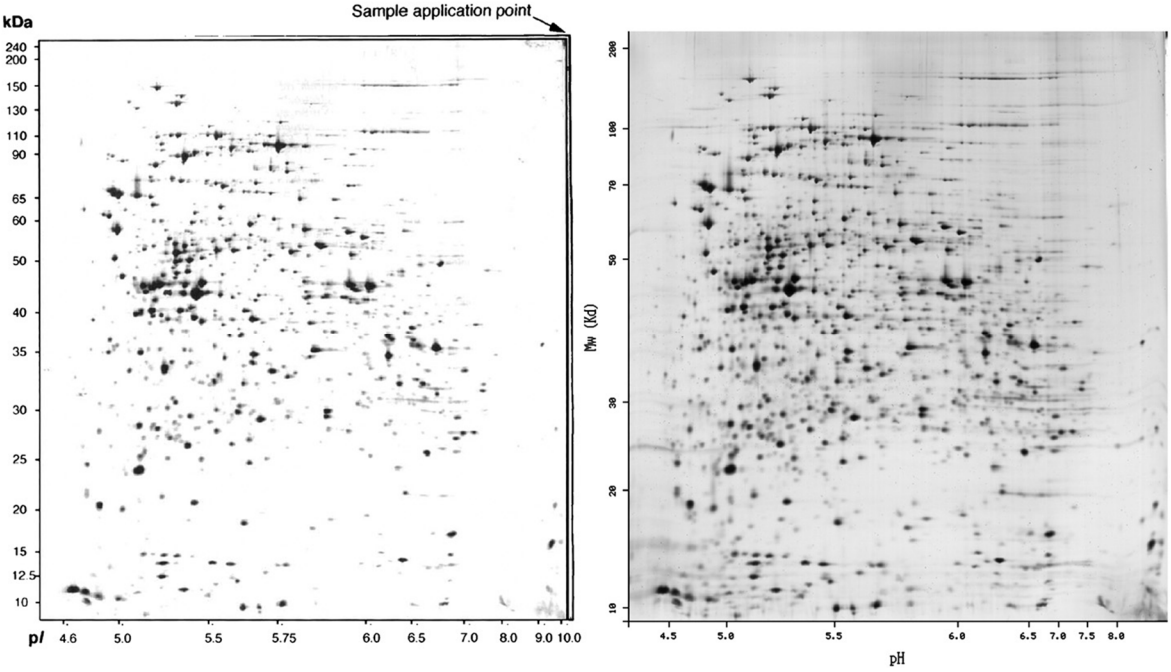

Figure I.3.2 | Left side: Silver-stained 2-D PAGE pattern of *E. coli* K-12 type extracts, strain W3110 and Right side: 3.5-10 silver-stained 2-D master gel of *E. coli* K-12 type extracts, strain W3110

Reproduced by permission from Pasquali et al. [1996]: Two-dimensional gel electrophoresis of *Escherichia coli* homogenates: The *Escherichia coli* SWISS-2DPAGE database. Electrophoresis 1996; 17: 547-555. Copyright Wiley-VCH Verlag GmbH & Co. KGaA and Reproduced by permission from Tonella et al. [1998]: '98 *Escherichia coli* SWISS-2DPAGE database update. Electrophoresis 1998; 19: 1960-1971. Copyright Wiley-VCH Verlag GmbH & Co. KGaA (Picture downloaded from SWISS-2DPAGE database), respectively

During the post-genomic era, proteome approaches have quickly acquired a special consideration (Aebersold & Mann, 2003; Pandey & Mann, 2000). Proteomic reports include now protein expression profiling of biological samples in a given physiological state to provide a large-scale characterization of all identified proteins including their post-translational alterations (structural proteomics) (Wasinger et al., 1995), comparison of protein expression levels in two or more physiological states (i.e. normal versus altered/pathological conditions) (comparative proteomics or quantitative proteomics) (Florens et al., 2002; Lasonder et al., 2002), protein–protein

interaction analysis (Gavin et al., 2002; Uetz et al., 2000), subcellular protein localization analysis (Huh et al., 2003) and definition of a biological role (functional proteomics) (Minshull et al., 2005). In comparative proteomic analyses, proteins from different biological conditions are quantitatively compared to obtain a better knowledge of the biological processes affecting their expression and/or in which they are involved. There are numerous techniques that have been developed to achieve this goal. Some of them are based on an extensive protein separation before identification. In this field, the two-dimensional electrophoresis (2-DE) is the most commonly applied separation methodology. Through the introduction of stable isotope labeling reagents, a specific quantitative protein evaluation in shotgun proteomic reports has been achieved (Goshe & Smith, 2003; Tao & Aebersold, 2003).

One of the main requirements of proteomics is the ability to relate the individual proteins resolved by 2-DE to the corresponding gene sequences. Several approaches can be made to identify the individual protein spots determined by 2-DE for bacteria. Early applications of 2-DE studying synthesis and gene expression in *E. coli* detected proteins present in the total cell lysates on the basis of their co-migration with purified proteins (Neidhardt et al., 1977; Pedersen et al., 1978). While valuable, these approaches are limited by the availability of either purified proteins or specific antibodies. The methods used to identify proteins are based on determining the protein's amino acid composition (Wilkins et al., 1996) or a partial amino acid composition (Lin & Ficht, 1995), or a peptide mass profile (Fountoulakis et al., 1998). These data can then be used to examine an appropriate nucleic acid or protein sequence database for a match to an existing gene sequence or homology to a linked gene or protein sequence. Proteomics have an important advantage once the genome of the microbe, and even the specific isolate, which is being investigated, is completely sequenced. This clearly advances the success rate of protein identification. The protein identification approaches used alone are not optimal for the identification of proteins recovered from poorly characterized bacteria. Though, several reports have proved that amino acid composition characterization provides a more accurate means of protein identification than peptide mass mapping (Cash, 2000).

The global analysis of cellular proteins is a fundamental area of research that is developing in the post-genome era and becoming an important tool in the research for complex biochemical processes, the discovery of new proteins and the investigation of protein–protein interactions (Osman et al., 2009). The application of proteomics provides major opportunities to elucidate disease mechanisms and to identify new diagnostic markers and therapeutic targets. This follows the great progress that is already along the same lines with bacterial pathogens characterization obtained by combining the genomic and proteomic technologies (Josic & Kovac, 2008). The concern for antibiotic resistance has led the scientific community to develop several tools and techniques in order to see the problem solved (Reinhart & Hartog, 2010; Werner et al., 2008). The investigation of the *E. coli* proteome can be classified generally into three phases: (i) the gel-based approaches, (ii) the non-gel-based approaches, and (iii) predictive proteomics (bioinformatics tools). The gel-based and non-

gel-based approaches are described as being based on the separation of complex protein mixtures in gel and non-gel matrices, respectively. However predictive proteomics cover functional proteomic reports achieved by using computational tools *in silico* (Han & Lee, 2006). These methods overlap in time, and their evolutions have resulted in an almost exponential increase in the number and quality of the identified protein spots over the past 3 decades (Tonella et al., 2001). Moreover increasingly complex separations have been developed to continue the progress. Recently, the *E. coli* proteome has been used as a standard method to evaluate and to validate new technologies and methodologies such as sample prefractionation, protein enrichment, 2-DE, protein detection, MS, combinatorial assays with n-dimensional chromatography and MS, and image analysis (Figure I.3.3).

Comparatively to the proteomes of other organisms, the *E. coli* proteome requires an exceptional model for several research needs based on the following advantages (Han & Lee, 2006; Lee & Lee, 2003): (a) the availability of public databases such as SWISS-PROT (http://www.expasy.ch/ch2d/) and NCBI (http://www.ncbi.nlm.nih.gov/), which contain rich data on the proteins and corresponding genes; (b) the presence of the *E. coli* SWISS-2DPAGE maps, which are based on a great deal of biochemical and biological data; and (c) the fact that the *E. coli* proteome is less complex than those of other organisms such as humans and plants, boasting smaller ORF products and less protein modification.

I.3.4. Proteomic methods

2-DE is currently the most widely used proteomic approach to analyze the protein composition of cells, tissues, or biofluids and it might even be called “classic” or “blue-collar” proteomics (Witzmann & Li, 2002). 2D gel electrophoresis achieves the separation of solubilized proteins in the first dimension according to their charge isoelectric point, followed by their separation in the second dimension by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to their relative molecular mass (Check, 2004). The principal strength of 2-DE consists of its particularly high-resolution power compared to other separation approaches and a good visualization of the obtained results. With the introduction of immobilized pH gradients (IPG) resolution, reproducibility and protein load capacity have been greatly improved (Görg et al., 1988). 2-DE can provide more than 10,000 detectable protein spots in a single gel run (Klose & Kobalz, 1995). Thus, proteins with post-translational modifications (PTMs), such as processing, phosphorylation and glycosylation, can be certainly found as separate spots on a 2D-E gel (Renzone et al., 2005). After tracing a spot within the gel, it may be excised, digested and subjected for analysis, typically by using a matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS (Klose & Kobalz, 1995). Nowadays, the 2-DE is the only method that can be usually used for parallel quantitative expression profiling of large sets of

complex protein mixtures such as whole cell lysates. This is in contrast to promising alternative technologies such as multidimensional protein identification technology, stable isotope labeling that have been developed recently. Furthermore, this approach gives us a map of proteins, which reflects modifications in the protein expression level, isoforms or post-translational changes.

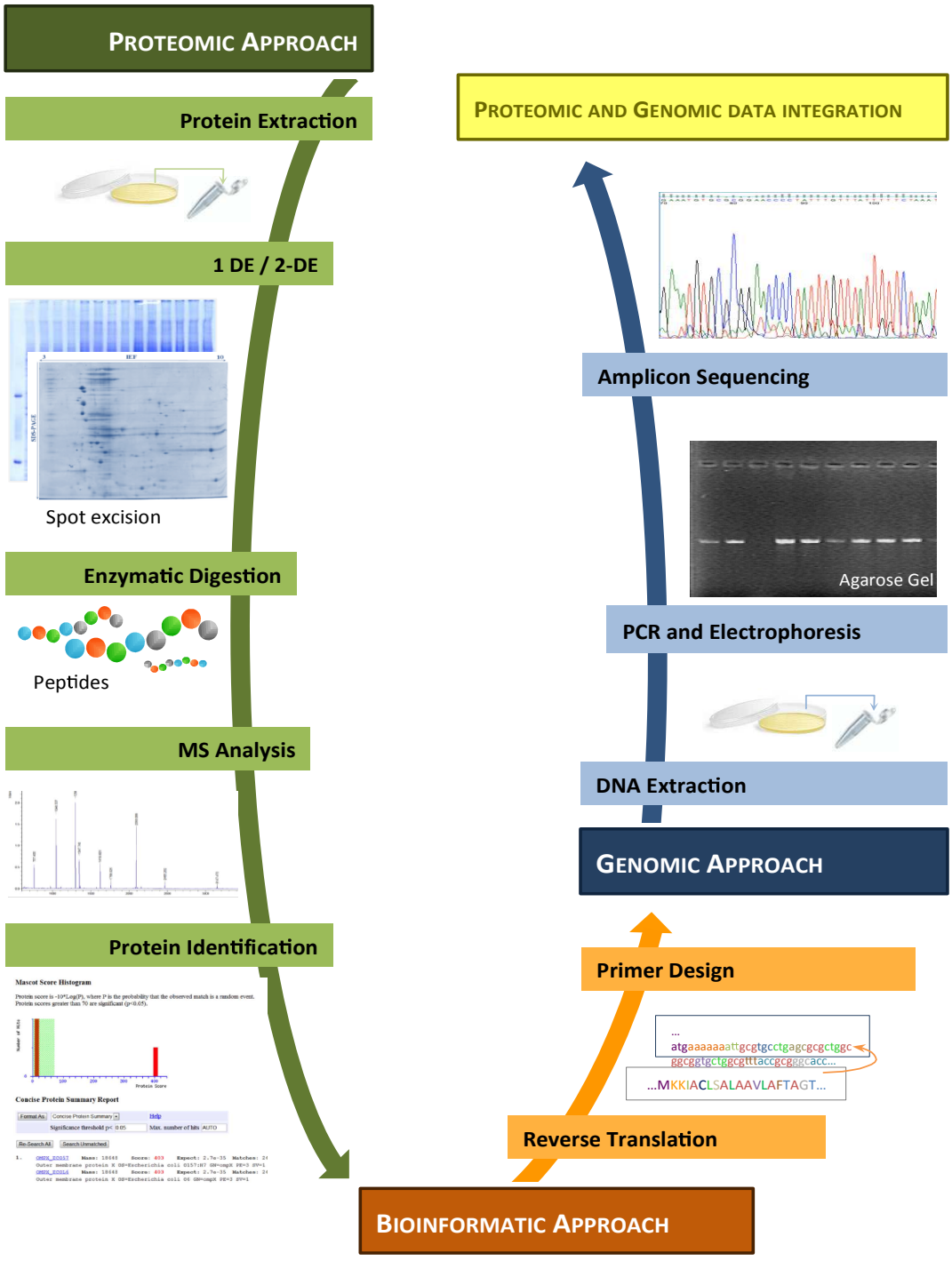

Figure I.3.3 | Typical proteomic workflow representing the classical gel-based approach to protein identification

On the other hand, liquid chromatography-tandem mass spectrometry based methods perform peptide analysis, where M*r* and pI information is lost, and where stable isotope labeling is required for quantitative analysis (Maurya Bharati et al., 2009).Also, the identification of the protein requires a mass/charge analyzer with good resolution. Currently, 2-DE technology with IPGs has overcome the previous limitations and time necessary for an extensive analysis confirming the need for new proteomic approaches and offering more information in a single, time-limited step. This statement is further supported by various studies, which showed that only the most abundant proteins in a sample were detected by this combined approach (Renzone et al., 2005).

A modern multiplexing technology for 2-DE, the fluorescent two dimensional differential gel electrophoresis (2D-DIGE), seems to overcome these limitations, since it is based on the use of a single gel which helps the simultaneous separation of multiple protein samples, followed by the independent visualization of each individual sample (Renzone et al., 2005). In fact, 2D-DIGE allows simultaneous detection and quantification of paired samples on the same gel, by covalently tagging samples at lysines by distinct and spectrally resolvable fluorescent derivatives of the cyanine dyes Cy2, Cy3 and Cy5 (Issaq & Veenstra, 2008). Posterior to the protein separation, it is important to stain effectively the obtained spots and to that effect there are different dyes and staining possibilities. Coomassie Brilliant Blue (CBB) is largely used in 2-DE experiments producing a high background staining of proteins present; however efforts are still made in order to ensure better results. Silver staining is an improvement to CBB guaranteeing a higher resolution but making it more difficult for the protein characterization by MS afterwards. On the other hand, fluorescent dyes are currently used in several studies, such as the protein labeling with cyanine applied in DIGE, alongside many other methods and procedures (Gauci et al., 2011).

A spot separated by 2-DE consists in theory of an almost homogeneous protein, and thus can be identified for content, following the digestion with a sequence-specific protease by using peptide mass fingerprinting (PMF) approaches. This is done typically using matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) mass spectrometers (Jungblut & Thiede, 1997; Yates, 1998). For peptide mass fingerprinting analysis, numerous search algorithms are now available. Peptide masses experimentally determined for a proteolytic digest by MALDI-TOF-MS analysis are compared to predicted masses from in silico digestions of all proteins present in a given database (Renzone et al., 2005). Gel-based proteomics participates in studying the expression of the virulence factors and in the interpretation of regulatory networks involved in bacteria virulence which is important to understand the strength of the virulence potential of pathogen and its interaction with the host (Engelmann & Hecker, 2009).

Restricted by these 2-DE limitations, discovering non-gel based alternatives relying on the separation of peptides rather than proteins is becoming more significant (Renzone et al., 2005). At a peptide level, 2D chromatographic approach designated multidimensional protein identification technology (MudPIT) has been considerably used to proteomics analyses (Gilmore & Washburn,

2010). It implicates digesting the whole protein sample and then separating the peptide mixture by one or two dimensions of chromatography prior to LC–MS analysis (Bjellqvist et al., 1982). MudPIT techniques, when applied to protein mixtures, result in a loss of intrinsic information about the intact proteins. This information, which permits the protein identification and a more complete characterization, disappears in this case because digestion precedes separation and MS analyses (Fujii et al., 2004). The separation of intact proteins by using liquid chromatography provides many advantages over the MudPIT method and the use of gel based approaches. Recently, liquid chromatography approaches that can be used for protein and peptide separations have been developed to handle proteomic analyses of complex samples (Neverova & Van Eyk, 2005).

By comparing gel-based and gel-free proteomics approaches, a common theme seems to appear. However gel-free approaches provide better proteome coverage because they have only found proteins with a sequence compatible with shotgun peptide sequencing analysis. Therefore, even if gel-free approaches have the potential for an easier automation, a complete proteome profiling still requires its complementation done by 2-DE based approaches (Renzone et al., 2005).

Increasing rates of bacterial resistance among common pathogens are threatening the effectiveness of even the most potent antibiotics and therefore the possibility of a pathogen resistant to all available antibiotic classes is one of the most serious problems concerning scientists today and a major public health apprehension (Siegel, 2008). The proteomic studies conducted in bacterial strains found in humans led to the identification of proteins associated to different strains of the same bacterial species. By the process of 2-DE combining IEF with IPGs and SDS-PAGE, proteins were separated by their isoelectric point and molecular weight. MALDI-TOF following trypsin digestion allowed to obtain the amino acid sequences of the separated proteins. Bioinformatics databases permitted the characterization and the identification of the proteins detected. NCBI database http://www.ncbi.nlm.nih.gov/ was used to compare peptide mass peaks from the MS readings.

Proteomics has developed and updated its use in today's scientific studies, revealing a great capacity to obtain information and to generate results that are not possible to achieve by any other areas of science. Facing such a development, the protein technologies can very much be the beginning of a new biomarker industry at the service of healthcare against antimicrobial resistance. Advances in mass spectrometry technology brought great improvements, especially for the capacity to define the fundamental cell components. Electrospray ionization (ESI) can be applied as following the molecular ions of interest which are formed directly from a solution applying a high electric field to the tip of a capillary through which the solution passes. When complete sequences are not available or where MALDI-TOF has failed to unequivocally identify a protein it is necessary to employ electrospray ionization tandem MS (ESI-MS/MS) to generate partial sequence information for database searching or for cloning experiments. ESI is an extremely sensitive technique, and moreover, low flow rate and reduced dimensionality variations such as microelectrospray and nanospray have been proven capable of excellent performance with remarkably high sensitivities.

The application of such a technology allows the efficient production of multiply charged ions, it permits the measurement of high-mass biopolymers with medium mass/charge ranges, and it also allows the application of multiple charges in a single molecule which improves fragmentation for structure identification (Chait, 2011).

The peptide molecular masses are combined with the sequence information from MS/MS data to perform the database search. SWISS-PROT non-redundant protein sequence database is used for all searches under *E. coli*. Bioinformatics platforms collaborate closely with mass spectrometry functioning at different levels like the alignment of reads at a reference sequence, *de novo* assembly or even genome annotation (Zhang et al., 2011). There are many search engines capable of reading the sequences detected by MS, and according to the studies' objective a proper bioinformatics tool must be selected (Jabbour et al., 2010; Mori, 2004).

To review what has been said up until now, the complete genomic sequences of many bacteria are publicly available at http://www.tigr.org/tigrscripts/ CMR2/CMRGenomes.spl. Based on a well annotated genomic sequence databases, it became possible to introduce large-scale technologies to identify every protein that a bacterium cell synthesizes. As a result, many bacterial protein 2D electrophoretic maps are today available and several are accessible through World-Wide-Web servers (for example, http://www.expasy.ch/ch2d/2d-index.html). These proteomic maps link a protein spot on a 2D gel to its corresponding nucleotide/protein sequence and to its knowledge of protein function and properties. Defining a 2D reference map is an important starting point for many physiological studies that may follow. By studying the expression levels of a multitude of proteins under several different growth conditions, specific markers of a particular cellular physiological state can be defined (Bonnedahl et al., 2009). However, to identify such a subset of proteins, it is important to prove the strict relationship between the expression level of a specific polypeptide product and a certain physiological condition.

I.3.5. Proteome mapping and the effects of stress environments on *E. coli*

The importance of the rapidity response to stresses is crucial for bacterial survival; experiments proved that bacteria developed defense mechanisms to fight against stress, which permit them to survive under severe conditions and unexpected environmental changes. These responses have important consequences for many of our interactions with bacteria. These consequences manifest themselves in at least four main areas (resistance to host defense systems during infection; reduction in the efficiency of lethal effects of chemical/physical treatments; failure to prevent pathogen growth in preserved and minimally processed foods; improvement of strains for industrial applications). It is important for the survival that the protein composition of a cell is continually adjusted to meet the challenges of changing environmental conditions (Renzone et al., 2005).

Therefore, bacteria respond to their environment with programmed changes in gene expression (cell division, DNA metabolism, housekeeping, membrane composition, transport, etc.) and act in order to increase the bacterial stress tolerance (Storz & Hengge-Aronis, 2000). Many are genes involved in the stress response. Usually regulators control several genes and occasionally disturb other regulators (VanBogelen & Neidhardt, 1990). Moreover, bacterial response to a particular stress can increase resistance to other stresses and can modify a number of other aspects of bacterial metabolism and the potential to interact with hosts. Discovering regulators and regulatory networks is important to control, predict or engineer bacterial behavior. Especially, the identification of crucial stress-related genes will expose targets for a specific management to stimulate or limit cellular growth. This is a development of valuable tools to screen for tolerant or sensitive strains and an evaluation of the fitness of the culture, which can help to improve procedures for growth in the culture (Renzone et al., 2005). As prokaryote bacteria, *E. coli* is known to be used as a model to study regulatory networks, protein function, and cell differentiation, since their genomes are comparatively small and the adaptation processes are less complex. Accordingly, proteomics has been successfully used to study bacterial protein expression under different growth conditions and/or various external stresses. In fact, proteomics can describe dynamic protein composition variations of a cell that is constantly adjusted to meet the challenges of the environmental changes (Renzone et al., 2005).

Mainly, the stress situations described before do not differ from the stresses induced by antibiotic attack. Antibiotics are a common encounter for various bacteria in their natural habitats, because many microorganisms produce them to suppress the growth of competitors. Therefore, even antibiotic classes that stem from purely synthetic approaches and that had never experienced bacteria during evolution can to a certain extent, mimic "natural" processes for which bacteria have involved regulatory mechanisms (Brotz-Oesterhelt et al., 2005). Numerous researches showed that there are five ways for a bacterium to acquire antimicrobial resistance: (1) resistance through reduced permeability to antimicrobial agents, which inhibits antimicrobial access to target sites; (2) active efflux of the antimicrobial from the cell; (3) mutation in the target site; (4) enzymatic alteration or degradation of the antimicrobial; and (5) acquisition of alternative metabolic pathways (McDermott et al., 2003). The elucidation of the molecular details of drug resistance is a very active area of research that crosses many disciplinary boundaries. This happens because understanding the mechanism(s) by which drug resistance develops leads to improvements in extending the efficacy of current antimicrobials (Cash, 2000; Howard et al., 2002). A recent study has shown that the cell envelope is an important target for drug-resistant mechanism(s) of many pathogens because many drugs need to rapidly diffuse into a cell to encounter their targets (Savage, 2001; Xu et al., 2006; Zhang et al., 2001).

During the past decade proteomic tools have been significantly optimized and have been used to investigate the differences in the protein expression profiles of cells grown under a broad spectrum of growth conditions and with different stress factors, including some antibiotics (Bandow

et al., 2003). The Microbial Genome Database (MBGD) works as a microbial genome platform for large-scale comparative genomics allowing the enhanced assignment of functional annotation in each bacterial species as well as serving as an interface of genome comparison based on phenotypic properties (Uchiyama et al., 2010). This platform is linked to other bioinformatic databases of genomic and proteomic interest, leading to cross-information in every sequence search made and therefore more precise information.

Comparative 2-DE proteomic approaches of *E. coli* isolates treated with several antibiotics or with temperature shock (VanBogelen & Neidhardt, 1990) showed that the heat and the cold shock responses could be represented by different sets of ribosome-targeting antibiotics. For example, streptomycin, puromycin, and kanamycin induced protein expression patterns that resemble the heat shock and stringent responses, while tetracycline, chloramphenicol, erythromycin, fusidic acid, and spiramycin invoked the cold shock and relaxed ribosomal responses (VanBogelen & Neidhardt, 1990). Through these discoveries, researchers have proposed that translational blocks induce heat shock-like or cold shock-like responses, indicating that the state of the ribosome or a ribosomal product may signal these responses (Han & Lee, 2006). The number of detected proteins in response to stress mechanisms represents only a small proportion of the predicted proteome, as only a certain number of genes may be induced and expressed under certain conditions (Coldham et al., 2006).

Our group has realized a proteomic evaluation of two *E. coli* isolates (C583 and C580), recovered from clinical human samples and which were carrying a plasmidic β-lactamase of class AmpC (CMY-2) and a TEM-52 enzyme, respectively. This was possible by using 2-DE and MALDI-TOF MS to accomplish the subsequent protein identification (Table I.3.1) (Pinto et al., 2011). *E. coli* strain C583 exhibited resistance to ampicillin, amoxicillin/clavulanic acid, cefoxitin, cefotaxime, ceftazidime, naladixic acid, ciprofloxacine, and trimethoprim sulfametoxazole and harbored *the intI*1 gene of class 1 integrons. A plasmidic β-lactamase of class AmpC (CMY-2) was detected in this strain. *E. coli* C580 presented resistance to ampicillin, cefotaxime, ceftazidime, naladixic acid, ciprofloxacin, and trimethoprim sulfametoxazole but did not contain class 1 integrons. This strain was proved to be a β-lactamase TEM-52 producer (Vinué et al., 2008). A comparative analysis among the strains was carried out and the protein expressions of *E. coli* strains were analyzed on 2-DE gels. The use of pH 3–10 IPG strips resulted in a well spread display of protein spots which permitted their safe and accurate excision and image identification. Proteins linked to nine different *E. coli* strains were identified in the samples of our C580 and C583 strains; namely proteins associated to three commensal strains (K12, O9:H4 and strain ATCC 8739/DSM 1576/Crooks), two uropathogenic (UTI89 and O6:K15:H31 (strain 536)), one avian pathogenic (O1:K1), one enterohemorrhagic (O157:H7), one enterotoxigenic (O139:H28 (strain E24377A)) and one virulent strain (O6). Related to the virulent *E. coli* strain O6 two proteins for *E. coli* isolate C583 (Trigger factor protein and Chaperone protein ClpB) and three proteins for *E. coli* isolate C580 (Trigger factor protein, Chaperone protein ClpB and Pyridoxine 5′-phosphate synthase protein) were

detected. As far as the enterohemorrhagic *E. coli* strain, two related proteins were identified in both *E. coli* isolate C583 (Putative flavoprotein and Serine hydroxymethyltransferase protein) and another two were found in *E. coli* C580 (Putative flavoprotein and Curved-DNA binding protein). The detection of proteins Malate dehydrogenase and GrpE protein linked with *E. coli* enterohemorrhagic strain O157:H7 in our human isolates was also noticed. Multiple antibiotic resistances in *E. coli* can be mediated by induction of the SoxS or MarA protein, triggered by oxygen radicals (in the *sox*RS regulon) or certain antibiotics (in the *mar*RAB regulon), respectively. Oxidative stress genes that are transcriptionally activated by both the *mar*RAB and *sox*RS systems include *sod*A (encoding a superoxide dismutase), zwf (encoding glucose-6-phosphate dehydrogenase [G6PD]), *fum*C (encoding a heat-stable fumarase), *soi-17/-19*, and the genes encoding two other oxidative stress proteins of unknown function (Ariza et al., 1995).

Usually, uropathogenic strains are involved in urinary tract infections (UTI) in humans, with UTI89 strain being related to the expression of adhesive organelles (known as pili) that interact with proteins of the urinary epithelial cells (Hunstad et al., 2005). Whereas proteins, previously characterized in the present review, as linked with three commensal strains were also found in *E. coli* isolates from both human feces samples: K12, O9:H4 and strain ATCC 8739/DSM 1576/Crooks. *E. coli* K12 is a well-known commensal strain with two major porines OmpC and OmpF, corresponding proteins of the external membrane (Hayashi et al., 2006). *E. coli* strain ATCC 8739/DSM 1576/Crooks differs from the K12 strain because of the presence of an insertion element IS1-13 associated to an upstream promoter and to the first 114 bp of OmpC. Therefore element IS1-13 only expresses OmpF (Pinto et al., 2011).

Dos Santos and co-workers (dos Santos et al., 2010) identified changes in the subproteome of a laboratory-derived piperacillin/tazobactam-resistant strain of *E. coli* (minimal inhibitory concentration [MIC] = 128 mg/L) as compared with its susceptible wild-type strain *E. coli* ATCC 25,922 (MIC = 2 mg/L). They have used 2-D fluorescence difference gel electrophoresis (2D-DIGE) followed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF MS). In the resistant strain, a total of 12 protein species were increased in abundance related to the wild-type strain, including those linked to bacterial virulence, antibiotic resistance and DNA protection during stress. This study shows overall changes in the subproteome of the piperacillin/tazobactam-resistant strain, reporting for the first time the potential role of a multidrug efflux pump system in *E. coli* resistance to piperacillin/tazobactam. From the numerous proteins identified, the chaperone clpB was increased in abundance in the resistant strain. This protein is part of a stress-induced multi-chaperone system in *E. coli*; it has participated in the recovery of the cell from heat-induced damage, and it has affected the processing of protein aggregates. In the resistant strain, there was a decreased abundance of protein species linked to the oxidative stress response and to the energy metabolism (Table I.3.1). Among the proteins that increased in abundance in the resistant strain was the outer membrane protein TolC. This protein combined with AcrAB, forms the

major typical multidrug efflux pump in *E. coli* (Sulavik et al., 2001). OmpA and OmpX were also increased in abundance in Ec-PTZ strain. OmpA, one of the most abundant and well-studied proteins of the cell wall of *E. coli*, is also important for bacterial virulence. The protein has been associated with: a) an invasion of brain micro-vascular endothelial cells by type 1 fimbrial modulation bore (Teng et al., 2006); b) an invasion of the intestinal epithelium (Venkitanarayanan & Nair, 2007); c) a biofilm formation (Orme et al., 2006); and d) a serum resistance in a neonatal rat model by interference with complement activation, inhibition of cytokine induction and the capability to multiply within macro-phages (Wooster et al., 2006). In case of OmpX, this protein presents similarity to OmpA. Another function that has been attributed to OmpX is the resistance to antimicrobials. A previous research (Stoorvogel et al., 1987) showed that the over-expression of this protein causes down regulation in the expression of the major pore proteins OmpF and OmpC, resulting in decreased susceptibility to beta-lactams. Resistance to piperacillin/tazobactam has been related to a reduced cell wall permeability of TEM-1-producing strains of *E. coli* (Marre et al., 1984). The several changes in protein abundance detected in the derived drug-resistant strain propose that resistant microorganisms may acquire molecular modifications in an effort towards adaptation to adverse environmental conditions (Justice et al., 2008; Linares-Rodriguez & Martinez-Menendez, 2005). This can have multiple consequences for various aspects of bacterial metabolism, which could be reflected in their virulence parameters (dos Santos et al., 2010).

Other comparative analysis by 2-DE of strains highly resistant to all antibiotics, except for penicillin, and strains resistant only to some classes of antibiotics were carried out (*E. coli* 111). This report points out the presence of (agmatinase) stress proteins, (alkyl hydroperoxide reductase, with antioxidant activity) (Roncada et al., 2009). A recent research (Lin et al., 2008) showed, for the first time, the outer membrane proteome (OM) of an *E. coli* strain resistant to nalidixic acid using proteomic methodologies. The OM proteins TolC, OmpT, OmpC and OmpW were found to be up-regulated, and FadL was down-regulated in the nalidixic acid-resistant *E. coli* strains. This study suggests that the TolC and OmpC proteins may play a more significant role in the control of nalidixic acid resistance more than other OM proteins identified. In addition, EnvZ/OmpR two-component system was found to be involved for the first time in the regulation of nalidixic acid resistance in *E. coli* (Table I.3.1).

Zhang and his investigation team (Zhang et al., 2008) confirmed by relying on two-dimensional gel electrophoresis subproteomics the presence of LamB, OmpC and TolC as important OM proteins for tetracycline resistance in *E. coli*. Meanwhile, our data suggest that functional validation is required to investigate whether the altered proteins play a direct or indirect role in the phenotypic changes.

A subproteomic approach (Li et al., 2008) was utilized to compare OM proteins of *E. coli* with streptomycin-resistance. TolC, OmpT and LamB were found up-regulated, and FadL, OmpW

and a Dps protein, with an unknown location, were down-regulated in the streptomycin-resistant *E. coli* strain. These changes at the level of protein expression were validated using Western blotting. The possible roles of the altered proteins involved in the streptomycin resistance were studied using genetic modified strains with the omission of these altered genes.

In the study of Li and co-workers (Li et al., 2007), comparative proteomic approaches were used to identify the sharedly altered outer membrane proteins (OM proteins) that are responsible for chloramphenical-resistant *E. coli*. Six different OM proteins and another protein with unknown location were determined to be sharedly chloramphenicol-resistant related proteins by using the 2-DE/MS, Western blotting and gene mutant methods. This resulted in the critical modification of TolC, OmpT, OmpC, and OmpW proteins and in the potential targets to design new drugs. Furthermore, a novel method of a specific antibody combating bacterial growth was developed relying on these OM proteins. This demonstrates that the enhancement of expression of an antibody target with antibiotic could be a very effective approach compared to using a drug alone, which highlights a potential way for the treatment of infection by using antibiotic-resistant bacteria (Li et al., 2007) (Table I.3.1). An applied proteomic methodology was used (Xu et al., 2006) to characterize the functional outer membrane proteins (Omps) of *E. coli* K-12 resistant to tetracycline and ampicillin in order to understand the universal pathways that form barriers for the antimicrobial agents. Different outer membrane proteins including TolC, OmpC and YhiU were related to the antibiotic resistance, while FimD precursors, LamB, Tsx, YfiO, OmpW, NlpB were first reported. It is known that some antimicrobial drugs can stimulate bacterial adhesion and toxin production and can interfere with the phagocytic process (Table I.3.1) (dos Santos et al., 2010; Lorian & Gemmel, 2005).

Table I.3.1 | Protein spots identification of 2-DE gels and MALDI-TOF sequencing results from different bacterial species and hosts

Spot	Accession Number	Protein description	Species	Protein Name	Protein MW/ PI	Biological process	Techniques used
118[a]	P63286	Chaperone protein clpB	*E. coli* O6	clpB	95526/5.37	Stress response	2 DE + MALDI – TOF/MS
119[a]	P0A6Z0	Chaperone protein dnaK	*E. coli* O157:H7	dnaK	69072/4.83	Stress response	
122[a]	Q8X4B4	Putative flavoprotein wrbA	*E. coli* O157:H7	wrbA	20821/5.91	DNA-dependent	
131[a]	A7ZN88	Chaperone protein hchA	*E. coli* O139:H28	hchA	31127/5.63	Stress response	
131[a]	A1ACB2	Chaperone protein hchA	*E. coli* O1:K1 / APEC	hchA	31186/5.42	Stress response	
131[a]	P59331	Chaperone protein hchA	*E. coli* O6	hchA	31217/5.62	Stress response	
181[a]	P0ABZ7	Chaperone protein surA	*E. coli* O6	surA	47254/6.48	Stress response	
218[a]	P78218	Dihydrofolate reductase type XV	*E. coli*	dhfrXV	17492/5.56	Antimicrobial resistance	

Spot	Accession Number	Protein description	Species	Protein Name	Protein MW/ PI	Biological process	Techniques used
100[b]	P63284	Chaperone protein clpB	*E. coli*	clpB	95697/5.37	Stress response	DIGE + MALDI – TOF/MS
132[b]	P02930	Outer membrane protein tolC	*E. coli*	tolC	53708/5.23	Antimicrobial resistance	
134[b]	P35340	Alkyl hydroperoxide Reductase subunit F	*E. coli*	ahpF	56484/5.47	Oxidative stress response	
140[b]	P0A910	Outer membrane protein A	*E. coli*	ompA	37292/5.6	Cell invasion	
151[b]	P0A917	Outer membrane protein X	*E. coli*	ompX	18648/5.3	Antimicrobial resistance	
153[b]	A7ZY70	DNA protection during DPS	*E. coli* O9:H4	dps	18684/5.7	Stress response	
1[c]	P02930	Outer membrane protein tolC	*E. coli*	tolC	53967/5.46	Antimicrobial resistance	2 DE + MALDI – TOF/MS + Western blotting
6[c]	P09169	Protease 7	*E. coli*	ompT	35540/5.76	Antimicrobial resistance	
8[c]	P06996	Outer membrane protein C	*E. coli*	ompC	40343/4.58	Antimicrobial resistance	
10[c]	P0A915	Outer membrane protein W	*E. coli*	ompW	22928/6.03	Antimicrobial resistance	
1[d]	P02943	Maltoporin	*E. coli*	lamB	47355/4.72	Antimicrobial resistance	2 DE + MALDI – TOF/MS + Western blotting
4[d]	P02930	Outer membrane protein tolC	*E. coli*	tolC	53967/5.23	Antimicrobial resistance	
6[d]	P06996	Outer membrane protein C	*E. coli*	ompC	40474/6.03	Antimicrobial resistance	
9[d]	P0A915	Outer membrane protein W	*E. coli*	ompW	25861/5.58	Antimicrobial resistance	
1[e]	P02930	Outer membrane protein tolC	*E. coli*	tolC	53967/5.46	Antimicrobial resistance	2 DE + MALDI – TOF/MS + Western blotting
5[e]	P10384	Long-chain fatty acid transport protein	*E. coli*	fadL	48742/5.09	Antimicrobial resistance	
6[e]	P09169	Protease 7	*E. coli*	ompT	35540/5.76	Antimicrobial resistance	
10[e]	P0A915	Outer membrane protein W	*E. coli*	ompW	22928/6.03	Antimicrobial resistance	
11[e]	P02943	Maltoporin	*E. coli*	lamB	49941/4.85	Antimicrobial resistance	
13[e]	P0ABT2	DNA protection during DPS	*E. coli*	dps	18564/5.72	Stress response	
1[f]	P02930	Outer membrane protein tolC	*E. coli*	tolC	53967/5.46	Antimicrobial resistance	2 DE + MALDI – TOF/MS + Western blotting
8[f]	P09169	Protease 7	*E. coli*	ompT	35540/5.76	Antimicrobial resistance	
13[f]	P06996	Outer membrane protein C	*E. coli*	ompC	40368/4.59	Cell invasion	
14[f]	P0A915	Outer membrane protein W	*E. coli*	ompW	22928/6.03	Antimicrobial resistance	
13[g]	P02930	Outer membrane protein tolC	*E. coli*	tolC	53967/5.23	Antimicrobial resistance	2 DE + MALDI – TOF/MS
17[g]	P02943	Maltoporin	*E. coli*	lamB	47355/4.85	Antimicrobial resistance	
25[g]	P06996	Outer membrane protein C	*E. coli*	ompC	40474/4.48	Cell invasion	
32[g]	P0A915	Outer membrane protein W	*E. coli*	ompW	25861/6.03	Antimicrobial resistance	

[a] Pinto et al. (Pinto et al., 2011), [b] dos Santos et al. (dos Santos et al., 2010), [c] Lin et al. (Lin et al., 2008), [d] Zhang et al. (Zhang et al., 2008), [e] Li et al. (Li et al., 2008), [f] Li et al. (Li et al., 2007), [g] Xu et al. (Xu et al., 2006)

Understanding the interaction of pathogenic microorganisms collaborate with their hosts to produce a clinical disease is one of the main goals for medical microbiology. One long-term practical outcome of this work will be the identification of novel virulence determinants that may serve as future targets for vaccine and drug development. Nowadays, recent molecular approaches permit researches to analyze these procedures in fine detail and, as discussed below, proteomics play a significant role in these developments.

The capability of pathogenic *E. coli* to cause disease in a susceptible host is determined by multiple factors acting individually or together at different stages during the infection. The diseases caused by a particular *E. coli* strain depend on the distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defenses. For example the 'Adherence' virulence factor, the Afa/Dr family which includes fimbrial adhesins AfaE-I, AfaE-III and Dr-II as well as the fimbrial Dr and F1845 adhesins. The Receptor is the DAF (decay-accelerating factor (CD55)), a cell-surface glycosylphosphatidylinositol-anchored proteins (GPI-APs) that normally protect cells from damage by complement system. GPI-APs may represent potential diagnostic and therapeutic targets in humans (Servin, 2005).

Proteomics has the potential synthesis and, by extension, the gene expression in a nonspecific manner, makes this approach a powerful tool to identify and to characterize the expression of *E. coli* virulence determinants. Using proteomic and microarray analyses, Dudley et al. (Dudley et al., 2006) identified for the first time that AggR activates the expression of chromosomal genes, including 25 contiguous genes (aaiA-Y), which are localized to a 117 kb pathogenicity island (PAI) inserted at pheU. Many of these genes have homologues in other Gram-negative bacteria and were recently proposed to constitute a type VI secretion system (T6SS). AaiC was identified as a secreted protein that has no apparent homologues within GenBank. Distribution studies indicated that aaiA and aaiC are commonly found in EAEC isolates worldwide, particularly in strains defined as typical EAEC. These data support the hypothesis that AggR is a global regulator of EAEC virulence determinants, and builds on the hypothesis that T6SS is an important mediator of pathogenesis. A comparative proteomics was used to investigate the expression of uropathogenic *E. coli* (UPEC) cytoplasmic proteins during growth in the urinary tract environment and a systematic disruption of central metabolic pathways to get a better knowledge of bacterial metabolism during infection (Alteri et al., 2009).

Many reports have investigated the genetics and the biochemistry of many different aspects of bacterial cell function (Alekshun & Levy, 2007; Gale et al., 1981; Walsh, 2003) in order to get a better knowledge of the molecular mechanisms of resistance to antibiotics (Table I.3.2). Moreover, the research of the antibiotic action and resistance has contributed considerably to our understanding of cell structure and function. Resistance processes are widely distributed in the microbial kingdom and have been well studied for a diversity of commensals (Davies, 1994) and pathogens; most can be disseminated by one or more distinct gene transfer mechanisms. A few of the resistance types that

illustrate the difficulties in conserving effective antibiotic activity in the face of the genetic and biochemical flexibility of bacteria deserve special attention. Bacteria acquire antimicrobial agent resistance genes through mobile elements, such as plasmids, transposons, and integrons (Prescott et al., 2000; Rubens et al., 1979). These elements have consequences in mutations of genes responsible for the antimicrobial agent uptake or binding sites or activation of portions of bacterial chromosomes (Alekshun & Levy, 2007; Hachler et al., 1991). The frequency at which an individual gene mutates to express an antimicrobial resistance phenotype is a complex phenomenon in which environment, cell physiology, bacterial genetics, and population dynamics each play their roles (Martinez & Baquero, 2000). The genes for β-lactamase enzymes are probably the most known genes internationally in distribution; random mutations of the genes encoding the enzymes have given rise to modified catalysts with increasingly extended spectra of resistance (Gniadkowski, 2008).

β-Lactam antibiotics can be classified into six different groups: penicillins, cephalosporins, carbapenems, cephamycins, monobactams, and β-lactamase inhibitors. The main cause of resistance to β-lactam antibiotics in Gram-negative bacteria is the production of β-lactamases, which are encoded chromosomally or on plasmids. The β-lactamases have the ability to inactivate β-lactams by hydrolyzing the four membered β-lactam rings.

Based on their primary structures, β-lactamases are divided into four classes (classes A, B, C and D) (Bradford, 2001). Genes encoding the β-lactamases of classes A, B and D are located on transferable plasmids or on the chromosome. Originally, class C β-lactamases were described as chromosomally encoded enzymes, but over the last 20 years several plasmid-encoded transferable class C enzymes have been identified (Philippon et al., 2002). In *E. coli*, resistance provided by class C β-lactamases can be plasmid encoded due to the overexpression of the chromosomal *amp*C gene. Another example of bacterial genetic jugglery comes from the recent appearance of a novel fluoroquinolone resistance, like nalidixic acid and ciprofloxacin. These are synthetic broad spectrum antibiotics with the capacity to inhibit bacterial DNA gyrase and topoisomerase IV implicated in breaking hydrogen bonds of DNA replication. Studies on the effects of sub-inhibitory concentrations of ciprofloxacin on gene expression in many microorganisms revealed dramatic changes in the transcriptome (Brazas & Hancock, 2005b). Such alterations enable the organism to adapt to its newer challenge and switch on mechanisms leading to its survival, and an example of this is the induction of SOS response by fluoroquinolone (Brazas & Hancock, 2005a).

Polymyxins are among the antimicrobial compounds highly used in bacteria such as *E. coli* and have been linked to mechanisms of adaptive resistance like the upregulation of the polysaccharides modification operon arn. As previously mentioned, polymyxins are also involved in the mechanism responsible for the adaptive resistance to cationic antimicrobials in low Mg^{2+} concentrations via systems PhoPQ and PamrAB. Peptide bacitracin and nisin are linked to the dysregulation of various genes of interest, such as encoding putative transporters and regulators, displaying a greater sensitivity towards antimicrobial peptides. Furthermore, transposons, elements

Table I.3.2 | Modes of action and resistance mechanisms of commonly used antibiotic for *E. coli* infections

Antibiotic classes	Examples of Antibiotics	Target	Mechanism(s) of action	Mode(s) of resistance
Beta-lactam (Penicillin)	Amoxicillin	Peptidoglycan biosynthesis	Inhibits protein synthesis and thus, bacterial growth, by binding to 30S and possibly 50S ribosomal subunits of susceptible bacteria	Hydrolysis, efflux, altered target
Beta-lactam (Monobactam)	Aztreonam	Peptidoglycan biosynthesis	Inhibits mucopeptide synthesis in the bacterial cell wall, thereby blocking peptidoglycan crosslinking. It has a very high affinity for penicillin-binding protein 3 (PBP-3) and mild affinity for PBP-1a	Hydrolysis, efflux, altered target
Beta-lactam (Carbapenem)	Meropenem	Peptidoglycan biosynthesis	Bactericidal broad-spectrum carbapenem antibiotic that inhibits cell wall synthesis	Hydrolysis, efflux, altered target
Beta-lactam (Carbapenem)	Imipenem and Cilastatin	Peptidoglycan biosynthesis	Cilastatin is a specific and reversible renal dehydropeptidase-I inhibitor. Since the antibiotic, imipenem, is hydrolyzed by dehydropeptidase-I, which resides in the brush border of the renal tubule, cilastatin is administered with imipenem to block the metabolism and thus the inactivation of imipenem so that antibacterial levels of imipenem can be attained in the urine. The drug also prevents the metabolism of leukotriene D4 to leukotriene E4 through the inhibition of leukotriene D4 dipeptidase	Hydrolysis, efflux, altered target
Beta-lactam (Cephalosporins)	Ceftriaxone	Peptidoglycan biosynthesis	Inhibits the mucopeptide synthesis in the bacterial cell wall. The beta-lactam moiety of Ceftriaxone binds to carboxypeptidases, endopeptidases, and transpeptidases in the bacterial cytoplasmic membrane. These enzymes are involved in cell-wall synthesis and cell division. By binding to these enzymes, Ceftriaxone results in the formation of defective cell walls and cell death	Hydrolysis, efflux, altered target
Beta-lactam (Penicillin and Beta-lactamase inhibitor)	Ampicillin and Sulbactam	Peptidoglycan biosynthesis	Drug combination of beta-lactamase inhibitor with ampicillin. Interferes with cell wall synthesis	Hydrolysis, efflux, altered target
	Piperacillin and Tazobactam	Peptidoglycan biosynthesis	Antipseudomonal penicillin plus beta-lactamase inhibitor. Inhibits biosynthesis of cell wall mucopeptide and is effective during stage of active multiplication	Hydrolysis, efflux, altered target

Antibiotic classes	Examples of Antibiotics	Target	Mechanism(s) of action	Mode(s) of resistance
Tetracycline	Doxycycline	Translation	Inhibits protein synthesis and thus, bacterial growth, by binding to 30S and possibly 50S ribosomal subunits of susceptible bacteria, blocking the binding of aminoacyl tRNA to the mRNA	Monooxygenation, efflux, altered target
Sulfonamide	Trimethoprim/ sulfamethoxazole	C1 metabolism	Inhibits bacterial growth by inhibiting synthesis of dihydrofolic acid	Efflux, altered target
Fluoroquinolone	Ciprofloxacin	DNA replication	Inhibits of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, strand supercoiling repair, and recombination	Acetylation, efflux, altered target
Fluoroquinolone	Levofloxacin	DNA replication	Inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase. Levofloxacin, like other fluoroquinolones, inhibits the A subunits of DNA gyrase, two subunits encoded by the *gyr*A gene. This results in strand breakage on a bacterial chromosome, supercoiling, and resealing; DNA replication and transcription is inhibited	Acetylation, efflux, altered target
Rifamycins	Rifampin	Transcription	Acts via the inhibition of DNA-dependent RNA polymerase, leading to a suppression of RNA synthesis and cell death	ADP-ribosylation, efflux, altered target

conferring a selectable phenotype flanked by two insertion sequences, are involved in horizontal gene transfer events between bacteria. Transposons are unique as they have the capability to transfer themselves from one genetic locus and to move to another, whether it is within the same bacteria or to another bacteria (Ochman & Jones, 2000).

Transposons can be transported through all of the methods mentioned above, namely conjugation, transformation, and transduction. Transposons play a significant role in the antimicrobial resistance development because they often contain gene sequences mediating antimicrobial resistance called integron gene sequences. Integrons are thought to play a significant role in the rapid dissemination of antimicrobial resistance among bacteria (Ochman & Jones, 2000). Although there is a wide branch of methodologies, genomics continues to evolve either by the increasing functionality of Real-time PCR and DNA pyrosequencing or by its growing connection to useful proteomic tools such as MALDI-TOF and bioinformatics (Garza-Ramos et al., 2009).

I.3.6. Outlook of proteomic biomarkers

Recent advances in biological and analytical sciences have led to an unprecedented interest in the discovery and the quantification of endogenous molecules, which serve as indicators for drug safety, mechanism of action, efficacy, and disease state progression. By allowing a better decision-making, these indicators, referred to as biomarkers, can dramatically improve the efficiency of drug discovery and development (Ackermann et al., 2006). The extraordinary developments made in proteomic technologies in the past decade have enabled investigators to search for biomarkers by scanning complex proteome samples and by using unbiased methods (Veenstra, 2007).

In the era of post-genome, proteomics has received a great deal of attention in the quest for protein biomarkers especially after it has been demonstrated that mass spectrometry (MS) revealed the ability to characterize a large number of proteins and also their post-translational modifications (PTM) in complex biological systems. In fact, there have been several technical advances in proteomics like the protein/antibody chips, the depletion of multiple high abundance proteins by affinity columns and the affinity enrichment of targeted protein analytes. As a matter of fact, proteomics has been broadly applied in a number of areas of science namely the deciphering of molecular pathogenesis of diseases, the characterization of innovative drug targets and the discovery of potential diagnostic and prognostic biomarkers all of these through the comparative analysis of expression levels and/or PTMs (Boja & Rodriguez, 2011). The development of protein microarray technology has also been helpful in the protein biomarkers research, providing simultaneous analysis of large numbers of different proteins within the same single experiment. This was done either to detect protein from capture molecules (forward phase) or arrays composed of proteins subsequently analyzed (reverse-phase) (Lin, 2010).

Advances in proteomics are closely related to the development of mass spectrometry-based platforms and technology, which allows the characterization of complex protein mixtures carrying different protein structure and composition. Within mass spectrometry, there are particular strategies that are viewed with greater interest by the scientists like the matrix-assisted laser desorption ionization–MS and the electrospray ionization–MS proteomics, 1-DE and 2-DE, gel-free shotgun in conjunction with liquid chromatography/tandem MS or even multiple reaction monitoring coupled tandem MS for quantitative proteomics (Chiou & Wu, 2011).

The next-generation sequencing technologies have demonstrated the ability to sequence DNA at unprecedented speed, thus enabling previously unconceivable scientific accomplishments and new biological applications. Shotgun sequencing technology was developed during the Human Genome Project, allowing the sequencing of large sections of DNA by previous enzymatic breaking of the DNA sequences into small fragments and cloning them into sequencing vectors in which cloned DNA fragments can be sequenced individually. Supporting the DNA sequencing, currently there are several platforms providing different systems for nucleotide and DNA sequencing, which leads to a wide development of this technology in general (Zhang et al., 2011).

The use of proteomics in the study of the antibiotic resistance in bacterial strains represents an important step to determine the metabolic pathways which result in antimicrobial behavior. The identification of proteins related to the antibiotic resistance represents valuable information on this problematic and the discovery of potential biomarkers related to the antibiotic resistance is a fundamental resource in order to fight against this healthcare menace. The preparation of 2-DE gels of drug resistant bacterial strains following elaborated phenotypic and genotypic profiles allows the identification and the characterization of the present proteins.

In the obtained proteomes, proteins previously identified in *E. coli* strains were found, some of them associated with pathogenic and virulent behavior. This has been possible only by dealing with a detailed proteomic approach and the combination between 2-DE and mass spectrometry (MALDI/TOF-TOF). The discovery of such proteins is a vital starting point to determine the genes and the proteins implicated in the biological processes responsible for the antibiotic resistance, thus taking a step forward in the struggle against this healthcare hazard.

I.3.7. Acknowledgements

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I.3.8. References

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CHAPTER II

AIMS OF THE STUDY

Aims of the study

The aim of the present dissertation was to investigate the ecological consequences of antimicrobial resistance in gastrointestinal commensal bacteria recovered from wild animals in Portugal, more specifically:

(i) To evaluate the prevalence of antimicrobial resistance in *E. coli* and enterococci strains.

(ii) To examine the occurrence and characteristics of extended-spectrum-β-lactamase (ESBL)-producing *E. coli* and vancomycin-resistant enterococci (VRE) strains.

(iii) To study the antimicrobial susceptibility profiles of antimicrobial-resistant *E. coli* and enterococci strains.

(iv) To understand the mechanisms of antimicrobial resistance in *E. coli* and enterococci strains and to identify the genes implicated in resistance and virulence through genomic tools.

(v) To elucidate the mechanisms of resistance in ESBL-*E. coli* and VRE strains and to identify marker proteins implicated in resistance and virulence through proteomic tools.

PART II

GENOMICS APPROACH

CHAPTER III

CHAPTER IV

CHAPTER III

GENOMIC CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT *E. COLI* AND ENTEROCOCCI ISOLATES OF WILD ANIMALS WITH NO ANTIMICROBIAL SELECTIVE PLATES

CHAPTER III

III.1. Radhouani H, Poeta, P, Igrejas G, et al. (2009) **Antimicrobial resistance and phylogenetic groups in isolates of *Escherichia coli* from seagulls at the Berlengas nature reserve.**

III.2. Radhouani H, Poeta P, Gonçalves A, et al. (2012) **Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of *Escherichia coli* and enterococci isolated from common buzzards (*Buteo buteo*).**

III.3. Radhouani H, Igrejas G, Gonçalves A, et al. (2013) **Antimicrobial resistance and virulence genes in *Escherichia coli* and enterococci from red foxes (*Vulpes vulpes*).**

III.4. Radhouani H, Igrejas G, Pinto L, et al. (2011) **Molecular characterization of antibiotic resistance in enterococci recovered from seagulls (*Larus cachinnans*) representing an environmental health problem.**

III.1. Antimicrobial resistance and phylogenetic groups in isolates of *Escherichia coli* from seagulls at the Berlengas nature reserve

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III.1.1. Abstract

Fifty-three faecal samples from yellow-legged gulls (*Larus cachinnans*) at the Berlengas nature reserve in Portugal were cultured on Levine agar plates not supplemented with antimicrobial agents, and one *Escherichia coli* colony was isolated and identified from each sample. The percentages of resistant isolates for each of the drugs were ampicillin (43.4%), tetracycline (39.6%), nalidixic acid (34%), streptomycin (32.1%), trimethoprim sulfamethoxazole (SXT) (26.4%), ciprofloxacin (18.9%), chloramphenicol (18.9%), gentamicin (7.5%), tobramycin (7.5%) amikacin (5.7%) and amoxicillin-clavulanic acid (1.9%). All the isolates were susceptible to cefoxitin, ceftazidime, cefotaxime, aztreonam and imipenem. The following resistance genes were detected: bla_{TEM} (17 of 23 ampicillin-resistant isolates), *tet*(A) and/or *tet*(B) (18 of 21 tetracycline-resistant isolates), *aad*A (12 of 17 streptomycin-resistant isolates), *cml*A (all chloramphenicol-resistant isolates), *aac*(3)-II with or without *aac*(3)-IV (all four gentamicin resistant isolates), and *sul*1 and/or *sul*2 and/or *sul*3 (all 14 SXT-resistant isolates). The *intI*1 gene was detected in 10 of 14 SXT-resistant isolates, and three of them also contained class 2 integrons; four different gene cassette arrangements were identified among class 1 integrons (*aad*A, *dfr*A1+*aad*A1, *dfr*A12+*orf*F+*aad*A2 and *sat*+*psp*+*aad*A2) and one among the class 2 integrons (*dfr*A1+*sat*+*aad*A1). Ninety per cent of the isolates were included in the A or B1 phylogenetic groups.

III.1.2. Introduction

Escherichia coli is a common inhabitant of the intestinal tract of animals and people (Sørum & Sunde, 2001; Tannock, 1995), and can be easily spread through water, soil and food. The microorganism can cause animal and human infectious diseases (Rosas et al., 2006; Sáenz et al., 2004) that require antimicrobial agents for treatment. However, in recent years *E. coli* has developed resistance to some of these agents that has been associated with failures in the treatment of these infectious diseases. Bacteria can be transferred between animals and people, including antimicrobial-resistant bacteria (Guardabassi et al., 2004), and for this reason it is important to analyse the phenotype and the mechanisms of antimicrobial resistance not only of pathogenic bacteria but also of commensal bacteria in the intestinal tract of people and animals. In fact, commensal bacteria such as *E. coli* can serve as reservoirs of resistance genes for potentially pathogenic bacteria (De Graef et al., 2004; Guardabassi et al., 2004; Moyaert et al., 2006; van den Bogaard & Stobberingh, 2000; Wray & Gnanou, 2000). Strains of *E. coli* can be classified into four main phylogenetic groups (A, B1, B2 and D) that were initially identified by the allelic variation of strains associated with enzymes that could be detected by multilocus enzyme electrophoresis (Herzer et al., 1990). More recently, a rapid and simple method for the determination of *E. coli* phylogenetic groups, based on a triplex PCR, has been reported (Clermont et al., 2000). Groups A and B1 are generally associated with commensal isolates, whereas in most cases enteropathogenic isolates are assigned to group D. It has been reported that isolates from phylogenetic group B2 more frequently accumulate extra-intestinal virulence factors (Duriez et al., 2001).

The Berlengas archipelago is a group of small islands off the Portuguese coast near the city of Peniche. It was one of the first protected areas in the world. Several species of seagulls nest on Berlengas Island, where their numbers have increased significantly in recent years and they are now considered a plague (Morais et al., 1995).

Analyses have been made of the antimicrobial resistance of commensal bacteria in the intestinal tract of domestic animals (Authier et al., 2006; Guardabassi et al., 2004; Moyaert et al., 2006; Normand et al., 2000; Sáenz et al., 2001; Sørum & Sunde, 2001), and in some wild animals (Caprioli et al., 1991; Cole et al., 2005; Costa et al., 2008b; Costa et al., 2006; Gilliver et al., 1999; Gionechetti et al., 2008; Kinjo et al., 1992; Muthukrishnan et al., 2011; Osterblad et al., 2001; Routman et al., 1985; Sayah et al., 2005; Skurnik et al., 2006). Important differences in the occurrence of antimicrobial resistance in isolates of *Enterobacteriaceae* from faecal samples have been detected in some of the latter studies (Gilliver et al., 1999; Osterblad et al., 2001), and some of them suggest that the resistance may be anthropogenic in origin (Sørum & Sunde, 2001). Poeta and others (Poeta et al., 2008) analysed the prevalence of extended-spectrum β-lactamases in selected *E. coli* isolates recovered on cefotaxime-supplemented agar plates from samples of faeces from Berlengas Island seagulls. The present paper describes investigations of the resistance phenotypes

and genotypes for a wide variety of antimicrobial agents and the phylogenetic groups in isolates of *E. coli* from the faeces of Berlengas Island seagulls, recovered in non-antibiotic supplemented agar plates, in order to obtain a deeper knowledge about the distribution of antimicrobial resistance genes in wild ecosystems.

III.1.3. Material and Methods

III.1.3.1. Samples and bacterial isolates

Fifty-three fresh faecal droppings were obtained from seagulls (*Larus cachinnans*) in different areas of Berlengas Island on five days during September 2007. Each sample was collected from the soil, transferred into a sterile plastic bag maintained at 4°C, and transported to the laboratory no longer than 24 hours after collection. Approximately 1 g of each sample was diluted in sterile 0.9 per cent saline solution, inoculated into Brain Heart Infusion (Oxoid) and incubated for 24 hours at 37°C; it was then spread on to eosin methylene blue agar (Levine; Oxoid), not supplemented with antimicrobial agents, and incubated for 24 hours at 37°C. One colony per sample, with typical *E. coli* morphology, was selected and identified by classical biochemical methods and by the API 20E system (BioMérieux).

III.1.3.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *E. coli* isolates was tested by the disk diffusion method on Mueller-Hinton Agar (Oxoid) following the recommendations of the Clinical and Laboratory Standards Institute (2007) (CLSI, 2007). A total of 16 antimicrobial agents were tested: ampicillin, amoxicillin-clavulanic acid, cefotaxime, cefoxitin, ceftazidime, imipenem, aztreonam, gentamicin, tobramycin, amikacin, streptomycin, tetracycline, trimethoprim-sulfamethoxazole (SXT), nalidixic acid, ciprofloxacin and chloramphenicol. *E. coli* ATCC 25922 was used as a quality control strain. The isolates with resistance to one or more antimicrobial agents were selected for the characterisation of their antimicrobial resistance genes.

III.1.3.3. Extraction of DNA

The isolates were cultivated on Brain Heart Agar (Oxoid) for 24 hours and later resuspended in 0.5 mL of sterile distilled water. The cells were lysed by heating at 95°C for 10 minutes and harvested by centrifugation at 12,000 rpm. The supernatant was used as the source of the template DNA for PCR.

III.1.3.4. Characterisation of antibiotic resistance genes

The presence of genes encoding TEM and SHV β-lactamases was studied by PCR in all the ampicillin-resistant isolates. The following genes were studied by PCR: *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(E) (in tetracycline-resistant isolates), *aad*A (in streptomycin-resistant isolates), *aac*(3)-II and *aac*(3)-IV (in gentamicin-resistant isolates), *aac*(6') (in amikacin-resistant isolates), *cml*A (in chloramphenicol-resistant isolates), and *sul*1*, sul*2 and *sul*3 (in SXT-resistant isolates). The presence of the *intI*1 and *intI*2 genes, encoding class 1 and 2 integrases, respectively, and the *qacEΔ1* gene were analysed by PCR in the SXT-resistant isolates. The variable region of class I integrons was studied by PCR. Positive and negative controls from the bacterial collection of the University of La Rioja, Spain, were used in all the assays. Table III.1.1 shows the primers and conditions used in the PCRs.

III.1.3.5. Detection of phylogenetic groups

The isolates of *E. coli* were assigned to one of the four main phylogenetic groups, A, B1, B2 and D, on the basis of the presence or absence of *chu*A*, yja*A or *tspE4.*C2 genes (Clermont et al., 2000). Table III.1.1 shows the primers used in these assays.

Table III.1.1 | Primers and annealing temperatures of PCR reactions used to detect antimicrobial resistant mechanisms

Primer name	Sequence 5´-3´	T (°C)	Amplicon size (bp)	Reference
TEM-F	ATTCTTGAAGACGAAAGGGC	60	1150	Sáenz et al., 2004
TEM-R	ACGCTCAGTGGAACGAAAAC			
SHV-F	CACTCAAGGATGTATTGTG	52	885	Pitout et al., 1998
SHV-R	TTAGCGTTGCCAGTGCTCG			
TetA-F	GTAATTCTGAGCACTGTCGC	62	937	Guardabassi et al., 2000
TetA-R	CTGTCCTGGACAACATTGCTT			
TetB-F	CTCAGTATTCCAAGCCTTTG	57	416	Guardabassi et al., 2000
TetB-R	CTAAGCACTTGTCTCCTGTT			
TetC-F	TCTAACAATGCGCTCATCGT	62	570	Guardabassi et al., 2000
TetC-R	GGTTGAAGGCTCTCAAGGGC			
TetD-F	ATTACACTGCTGGACGCGAT	57	1104	Guardabassi et al., 2000
TetD-R	CTGATCAGCAGACAGATTGC			
TetE-F	GTGATGATGGCACTGGTCAT	62	1179	Guardabassi et al., 2000
TetE-R	CTCTGCTGTACATCGCTCTT			
AadA-F	GCAGCGCAATGACATTCTTG	60	282	Sáenz et al., 2004
AadA-R	ATCCTTCGGCGCGATTTTG			
Aac(3)-II-F	ACTGTGATGGGATACGCGTC	60	237	Van De Klundert & Vlieegenthart, 1993
Aac(3)-II-R	CTCCGTCAGCGTTTCAGCTA			
Aac(3)-IV-F	CTTCAGGATGGCAAGTTGGT	60	286	Van De Klundert & Vlieegenthart, 1993
Aac(3)-IV-R	TCATCTCGTTCTCCGCTCAT			
Aac(6´)-F	TTGCGATGCTCTATGAGTGGCTA	55	482	Park et al., 2006
Aac(6´)-R	CTCGAATGCCTGGCGTGTTT			
CmlA-F	TGTCATTTACGGCATACTCG	55	455	Sáenz et al., 2004
CmlA-R	ATCAGGCATCCCATTCCCAT			
Sul1-F	TGGTGACGGTGTTCGGCATTC	63	789	Mazel et al., 2000
Sul1-R	GCGAGGGTTTCCGAGAAGGTG			

Primer name	Sequence 5´-3´	T (°C)	Amplicon size (bp)	Reference
Sul2-F	CGGCATCGTCAACATAACC	50	722	Maynard et al., 2003
Sul2-R	GTGTGCGGATGAAGTCAG			
Sul3-F	GAGCAAGATTTTTGGAATCG	51	792	Perreten & Boerlin, 2003
Sul3-R	CATCTGCAGCTAACCTAGGGCTTTGG			
IntI1-F	GGGTCAAGGATCTGGATTTCG	62	483	Mazel et al., 2000
IntI1-R	ACATGGGTGTAAATCATCGTC			
IntI2-F	CACGGATATGCGACAAAAAGGT	62	788	Mazel et al., 2000
IntI2-R	GTAGCAAACGAGTGACGAAATG			
VR1-F	GGCATCCAAGCAGCAAG	55	Variable	Sáenz et al., 2004
VR1-R	AAGCAGACTTGACCTGA			
QacEΔ1-F	GGCTGGCTTTTTCTTGTTATCG	62	287	Mazel et al., 2000
QacEΔ1-R	TGAGCCCCATACCTACAAAGC			
Chua-F	GACGAACCAACGGTCAGGAT	55	279	Clermont et al., 2000
Chua-R	TGCCGCCAGTACCAAAGACA			
YjaA-F	TGAAGTGTCAGGAGACGCTG	55	211	Clermont et al., 2000
YjaA-R	ATGGAGAATGCGTTCCTCAAC			
TspE4.C2-F	GAGTAATGTCGGGGCATTCA	55	152	Clermont et al., 2000
TspE4.C2-R	CGCGCCAACAAAGTATTACG			

III.1.4. Results

III.1.4.1. Percentages of antimicrobial resistance and resistance phenotypes

Table III.1.2 shows the percentages of the 53 isolates that were resistant to each of the antimicrobials tested. The percentages of *E. coli* that were resistant to either streptomycin, nalidixic acid, ampicillin or tetracycline ranged from 32.1 to 43.4 per cent, the percentages resistant to chloramphenicol, ciprofloxacin or SXT ranged from 18.9 to 26.4 per cent and the percentages resistant to amoxicillin-clavulanic acid, amikacin, gentamicin or tobramycin ranged from 1.9 to 7.5 per cent. All the isolates were susceptible to cefoxitin, ceftazidime, cefotaxime, aztreonam and imipenem. The phenotypes of resistance of the 53 isolates are shown in Table III.1.3. Twenty-nine of them were resistant to one or more of the antimicrobial agents, and 15 were resistant to at least five different families of antimicrobial agents.

Table III.1.2 | Number and percentages of the 53 isolates of *E. coli* from faecal samples from yellow-legged gulls that were resistant to different antibiotic agents

Antibiotic	Antibiotic-resistant isolates number (%)
Ampicillin	23 (43.4)
Amoxicillin-clavulanic acid*	1 (1.9)
Gentamicin	4 (7.5)
Tobramycin	4 (7.5)
Amikacin	3 (5.7)
Streptomycin	17 (32.1)
Tetracycline	21 (39.6)
Trimethoprim-sulfamethoxazole	14 (26.4)
Nalidixic acid	18 (34)
Ciprofloxacin	10 (18.9)
Chloramphenicol	10 (18.9)

*Isolates in the resistant and intermediate category are included in this section

Table III.1.3 | Phenotypes of antibiotic resistance detected among the 53 isolates of *E. coli* recovered from yellow-legged gulls

Phenotype of antibiotic resistance	Number of isolates
AMP	2
STR	1
TET	1
AMP-NAL	1
AK-STR	1
TET-NAL	1
AMP-STR-TET	1
AMP-TET-NAL	1
TOB-STR-NAL	1
AK-TET-NAL	1
AMP-TET- NAL-CIP	1
AMP- STR-TET-NAL	1
STR-TET-SXT-CHL	1
AMP-GEN-TOB-STR -TET	1
AMP-STR-TET-SXT-CHL	2
AMP-STR-TET-NAL-SXT	2
AMP-TET-NAL-CIP- CHL	1
AMP-NAL-CIP- SXT-CHL	1
AMP-STR -TET-CIP-SXT-CHL	1
AMP-STR-TET- NAL-CIP- SXT	1
AMP-AMC-STR -TET-NAL-SXT	1
AMP-GEN-TOB- NAL-CIP- SXT-CHL	1
AMP-AK-TET-NAL-CIP -SXT-CHL	1
AMP-STR-TET-NAL -CIP-SXT-CHL	1
AMP-GEN-STR-TET-NAL-CIP -SXT-CHL	1
AMP-GEN-TOB-STR-TET-NAL-CIP -SXT	1
Susceptible	24

AK Amikacin, AMC Amoxicillin-clavulanic acid, AMP Ampicillin, ATM Aztreonam, CAZ Ceftazidime, CHL Chloramphenicol, CIP Ciprofloxacin, CTX Cefotaxime, GEN Gentamicin, NAL Nalidixic acid, STR Streptomycin, SXT Trimethoprim-sulfamethoxazole, TET Tetracycline, TOB Tobramycin

III.1.4.2. Mechanisms of antibiotic resistance

The resistance genes detected among the antimicrobial-resistant isolates of *E. coli* are shown in Table III.1.4. The presence of β-lactamase genes was investigated in all 23 ampicillin-resistant isolates and a bla_{TEM} gene was detected in 17 of them; the bla_{SHV} gene was not found in any of these isolates. The *aac*(3)-II or *aac*(3)-IV genes, encoding an aminoglycoside acetyl transferase that modifies gentamicin and tobramycin, were detected in the four gentamicin-resistant isolates. In addition, the *aad*A gene, encoding an aminoglycoside adenyl transferase that modifies strepto mycin, was detected in 12 of the 17 streptomycin-resistant isolates. All the amikacin-resistant isolates were negative for the presence of the *aac*(6') gene. The *tet*(A) or *tet*(B) genes, associated with an active efflux system, were identified in 18 of the 21 tetracycline-resistant isolates, and both genes were detected in one of them. The *cml*A gene was found in all 10 of the chloramphenicol-resistant isolates. A total of 14 *E. coli* isolates showed the phenotype SXT-resistant and the *sul*1 and/or *sul*2 and/or *sul*3 genes were detected in all of them. Six of these isolates carried both the *sul*1 and *sul*2 genes, and one isolate carried both the *sul*2 and *sul*3 genes.

Table III.1.4 | Genes of resistance detected among the 29 antimicrobial-resistant isolates of *E. coli* recovered from yellow-legged seagulls

Phenotype of resistance	Number of isolates	Genes detected by PCR and sequencing	
		Gene	Number of isolates
Ampicillin	23	bla_{TEM}	17
Gentamicin	4*	*aac*(3)-II	2
		aac(3)-II+*aac*(3)-IV	2
Amikacin	3	*aac*(6´)	0
Streptomycin	17	*aad*A	12
Tetracycline	21	*tet*(A)	15
		tet(B)	2
		tet(A)+*tet*(B)	1
Trimethoprim-sulfamethoxazole	14	*sul*1	4
		*sul*2	2
		*sul*1+*sul*2	6
		*sul*1+*sul*3	1
		*sul*2+*sul*3	1
Chloramphenicol	13	*cml*A	10

* Three of these isolates were also resistant to tobramycin

The *intI*1 gene encoding class 1 integrase was detected in 10 of 14 SXT-resistant isolates, and eight of them amplified the class 1 integron variable region. The gene cassette arrangements detected among the *intI*1-positive isolates were as follows: *dfr*A1+*aad*A1 (four isolates), *sat*+*psp*+*aad*A2 (one isolate), *dfr*A12+*orf*F+*aad*A2 (one isolate), *aad*A and *dfr*A1+*aad*A1 (one isolate), *aad*A and *dfr*A12+*orf*F+*aad*A2 (one isolate) and unknown gene cassettes in the variable region (two isolates). Three of the 10 *intI*1-positive isolates also carried the *intI*2 gene, specific to class 2 integrons, and the *dfr*A1+*sat*+*aad*A1 gene cassette arrangement was identified in their variable regions.

III.1.4.3. Phylogenetic group

Table III.1.5 shows the phylogenetic groups to which the 29 antimicrobial resistant isolates belonged in relation to the number of antimicrobial agents to which they were resistant. Most of the isolates belonged to phylogenetic groups A (11 isolates) or B1 (15 isolates), and only two isolates were classified into the B2 group (both resistant to one or two antibiotics), and one isolate into the D group (a multiresistant isolate).

III.1.5. Discussion

There is little information about the susceptibility to antimicrobial agents of isolates of *E. coli* from healthy wild animals, even less about seagulls, and most of the studies have been limited to a small number of animals. The percentages of the isolates that have been observed to be resistant have varied with the country from which the animals came. In the UK, (Gilliver et al., 1999) observed high levels of resistance in faecal *Enterobacteriaceae* from wild rodents that had not been

Table III.1.5 | Phenotypes of resistance and phylogenetic groups detected among the 29 antibiotic-resistant isolates of *E coli* recovered from yellow-legged seagulls

Number of antibiotics to which *E. coli* were resistant	Number of isolates	Phylogenetic group			
		A	B1	B2	D
1	4	1	2	1	0
2	3	1	1	1	0
3	4	3	1	0	0
4	3	1	2	0	0
5	7	3	4	0	0
6	3	0	3	0	0
7	3	1	2	0	0
8	2	1	0	0	1
Total resistant isolates	29	11	15	2	1

in contact with antibiotics, whereas in Finland, (Osterblad et al., 2001) did not detect resistance among *Enterobacteriaceae* from wild moose, deer and voles. It has been suggested that there may be a correlation between the level of antimicrobial resistance in faecal bacteria from animals and the level of contact of these animals with people (Skurnik et al., 2006). The seagulls nest on the nature reserve of Berlengas but they usually fly to the Peniche coast where they may eat the remains of human food; this might explain the high rates of antimicrobial resistance found in the faecal isolates of *E. coli* from them, although they were lower than those reported for *E. coli* from food-producing animals (Kang et al., 2005; Sáenz et al., 2001; Van De Klundert & Vlieegenthart, 1993). The close contact with people and the possible heavy use of antimicrobial agents in food-producing animals may explain these differences.

The highest levels of resistance were observed for ampicillin and tetracycline, followed by nalidixic acid, streptomycin and SXT. Similar results were obtained in a study of faecal isolates of *E. coli* from wild animals in several nature parks in Portugal (Costa et al., 2008b), and faecal *E. coli* from food-producing animals in other European countries (Aarestrup, 1999; Guerra et al., 2003; van den Bogaard & Stobberingh, 2000).

The TEM β-lactamase was the most frequent mechanism of ampicillin resistance among the isolates (73.9 per cent), and it has also been detected in ampicillin-resistant isolates of *E. coli* recovered from food, animals and people (Briñas et al., 2002). This gene has also been detected in the majority of isolates from healthy pets in Portugal (Costa et al., 2004; Costa et al., 2008a). Similar results were obtained in isolates from the rumen of sheep in Slovakia (Malik et al., 2004), and also in almost all the strains from black-headed gulls in the Czech Republic (Dolejska et al., 2007).

The detection of *tet*(A) and/or *tet*(B) genes in almost 86 per cent of the tetracycline-resistant isolates indicates that the main mechanism of tetracycline resistance in the isolates of *E. coli* from seagulls was by active efflux. There was a predominance of the *tet*(A) gene (76.2 per cent). To date, eight different tet genes for efflux proteins have been sequenced in Gram-negative bacteria-*tet*(A-E), (G), (H) and (J) (Schwarz et al., 2001).

Ten per cent of the isolates of *E. coli* contained class 1 integrons, and 6 per cent contained both class 1 and class 2 integrons; 34 per cent of the resistant isolates contained integrons.

Guerra and others (Guerra et al., 2003) obtained similar results in isolates of *E. coli* from cattle, pigs and poultry. This high prevalence of integrons among commensal *E. coli* from seagulls is a cause for concern because this genetic structure is very efficient for the acquisition of antimicrobial resistance genes, which could be transmitted to other bacteria by mobile elements such as plasmids and transposons. Integrons appear to occur not only among clinical isolates of *E. coli* but also among commensal strains, even in wild animals.

The combination of two gene cassettes (*dfr*A1+*aad*A1) encoding resistance to trimethoprim and streptomycin was identified in five of the integron-positive isolates. This gene combination has been frequently detected among resistant isolates of *E. coli* from healthy animals and food products (Sáenz et al., 2004; Sunde, 2005), and also in healthy pets (Costa et al., 2004; Costa et al., 2008a). One additional *intI*1-positive isolate contained the combination *dfr*A12+*orf*F+*aad*A2 gene cassettes inside the class 1 integron variable region, and lacked the *sul*1 gene on the integron 3'-conserved region, usually included in class 1 integrons (Mazel et al., 2000). Nevertheless, defective class 1 integrons, lacking the 3'-conserved region, have been reported (Sáenz et al., 2004; Sunde, 2005).

The higher prevalence of A and B1 phylogroups compared with B2 and D phylogroups among the isolates could be explained by their faecal origin. The B2 isolates were resistant to only one or two antimicrobial agents, whereas the isolates of the other groups were resistant to more, particularly the isolate of group D that was resistant to eight antimicrobial agents.

Genes implicated in resistance to antimicrobial agents of different families (β-lactams, aminoglycosides, tetracycline, trimethoprim and sulfonamides) were detected in high proportions of faecal isolates of *E. coli* from the seagulls, although they were in general lower than those previously observed by other authors in food-producing animals (Sáenz et al., 2001), probably as a result of a lower antimicrobial pressure in the seagulls. Some of the resistance genes occurred in commensal bacteria of the seagulls, indicating that even though they do not naturally come into contact with antimicrobial agents, these animals can be colonised by resistant *E. coli* and can act as a reservoir of resistance genes. Because they often eat the remains of human food and can fly over long distances, these free-living birds may facilitate the circulation of resistant strains in different environments (Gionechetti et al., 2008). More studies are needed to investigate the evolution of antimicrobial resistance among faecal isolates of *E. coli* in different ecosystems.

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III.2. Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of *Escherichia coli* and enterococci isolated from common buzzards (*Buteo buteo*)

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III.2.1. Abstract

A total of 36 *Escherichia coli* and 31 enterococci isolates were recovered from the 42 common buzzard faecal samples. Resistances in *E. coli* present high percentages of streptomycin and tetracycline. The following resistance genes were detected: bla_{TEM} (20 of 22 ampicillin-resistant isolates), *tet*(A) and/or *tet*(B) (16 of 27 tetracycline-resistant isolates), *aad*A1 (8 of 27 streptomycin-resistant isolates), *cml*A (3 of 15 chloramphenicol-resistant isolates), *aac*(3)-II with/without *aac*(3)-IV (all 7 gentamicin-resistant isolates), *sul*1 and/or *sul*2 and/or *sul*3 (all 8 sulfamethoxazole-trimethoprim–resistant (SXT) isolates). *IntI*1 and *IntI*2 genes were detected in four SXT-resistant isolates. The virulence-associated genes type-1 fimbriae (*fim*A), P fimbriae (*pap*C) and aerobactin (*aer*) were detected in 61.1%, 13.8% and 11.1% of the isolates, respectively. The isolates belonged to A (47.2%); B1 (8.3%); B2 (13.9%), and D (30.5%) phylo-groups. For the enterococci isolates, *E. faecium* was the most prevalent specie (48.4%). High percentages of tetracycline and erythromycin resistances were found among our isolates (87% and 81%, respectively). Most of tetracycline-resistant strains carried the *tet*(M) and/or *tet*(L) genes. The *erm*(B) gene was detected in 80% of erythromycin-resistant isolates. The *vat*(D) and/or *vat*(E) were found in 9 of the 17 quinupristin-dalfopristin-resistant isolates. The enterococcal isolates showing high level resistance (HLR) for kanamycin, HLR for gentamycin and HLR for streptomycin contained the *aph*(3′)-IIIa, *aac*(6′)-*aph*(2″) and *ant*(6)-Ia genes, respectively. Our report reveals that common buzzards seem to represent an important reservoir or at least source of multiresistant *E. coli* and enterococci isolates, and consequently may represent a considerable hazard to human and animal health by transmission of these isolates to waterways and other environmental sources via their faecal deposits.

III.2.2. Introduction

Microbial resistance to antibiotics is a worldwide problem in human and veterinary medicine. Commonly, it is usual that the principal risk factor for the increase of this situation is an extensive use of antibiotics that leads to the dissemination of resistant bacteria and resistance genes in animals and humans (van den Bogaard & Stobberingh, 2000). The appearance of multiresistant bacteria of human and veterinary origin is probably accompanied by co-contamination of the environment apparently leading to a great health concern (Grobbel et al., 2007).

Bacteria may present resistance to antibiotics under selective pressure, but they may also acquire antibiotic resistance determinants without direct exposure to an antibiotic through horizontally mobile elements including conjugative plasmids, integrons and transposons (Middleton & Ambrose, 2005). These mobile elements can simply transfer antibiotic resistance genes from one bacterium to another (Coque et al., 2008). The bacteria of the normal flora of the gut, such as *Escherichia coli* and enterococci, can easily acquire and transfer resistance genes. These commensal bacteria, which constitute a reservoir of resistance genes for pathogenic bacteria, can thus be used as indicators of changes in antimicrobial resistance (Caprioli et al., 2000).

Antibiotic resistance in the faecal indicator bacteria could have several consequences. *E. coli* and enterococci have become more efficient human nosocomial pathogens (Jett et al., 1994), since they have developed increased antibiotic resistance.

The common buzzard (*Buteo buteo*) is a medium to large bird of prey, with a geographical distribution that covers most of Europe and also it extend into Asia. As a great opportunist, it is well adapted to a varied diet of pheasant, rabbit, other small mammals, snakes and lizards and can often be seen walking over recently ploughed fields looking for worms and insects (IUCN, 2010).

In addition to the currently common detection of multiresistant bacteria in areas with high human density (Cole et al., 2005), their emergence in more remote areas like high mountain regions is even more alarming (Dolejska et al., 2007). Although wild birds have only rare contact with antimicrobial agents, in disagreement with the existence of direct selective pressure, they can be contaminated or colonized by resistant bacteria. Water contact and acquisition via food seem to be major aspects of transmission of resistant bacteria of human or veterinary origin to wild animals (Cole et al., 2005). Wild birds in general may therefore represent reservoirs of resistant bacteria and genetic determinants of antimicrobial resistance (Dolejska et al., 2007).

Monitoring the prevalence of resistance in indicator bacteria such as faecal *Escherichia coli* and enterococci in different populations, animals, patients and healthy humans, makes it feasible to compare the prevalence of resistance and to detect transfer of resistant bacteria or resistance genes from animals to humans and vice versa (Martel et al., 2001). However, few reports of the level of antimicrobial resistance in *Escherichia coli* and enterococci of wild animal have been published (Nulsen et al., 2008; Poeta et al., 2007b; Poeta et al., 2005b; Radhouani et al., 2009; Silva et al., 2010).

The aim of the present study was to analyze the prevalence of antimicrobial resistance and the mechanisms implicated in faecal *E. coli* isolates and *Enterococcus* species of common buzzards in Portugal.

III.2.3. Methods

III.2.3.1. Samples and bacteria

Forty-two faecal samples from common buzzards (*Buteo buteo*) of Portugal were recovered from September 2007 to February 2008. These faecal samples were obtained during September 2007 to February 2008. All the faecal samples were collected individually from each animal and obtained in collaboration with Center of Collecting, Welcome and Handling of Wild Animals (CRATAS), located in the Trás-os-Montes and Alto Douro University (Portugal), which receives injured animals found in its natural environments. None of the animals had been fed previously by humans or had received antibiotics. The common buzzard is fairly well distributed throughout the Portuguese territory, being the only species of bird of prey found in all regions of the country. The majority of the birds came from the centre and north of the country. For *E. coli* isolation, samples were seeded in Levine agar plates and incubated 24 h at 37 °C. Colonies with typical *E. coli* morphology were selected and identified by classical biochemical methods (Gram, catalase, oxidase, indol, methyl-red-voges-proskauer, citrate and urease tests), and using the API 20E system (BioMérieux).

For enterococcal isolation, faecal samples were diluted and sampled in Slanetz–Bartley agar plates and were incubated for 48 h at 37 °C. Colonies with typical enterococcal morphology (one per sample) were identified by cultural characteristics, Gram-staining, catalase test and bile-aesculin reaction and by biochemical tests using the API ID20 Strep system (BioMérieux, La Balme Les Grottes, France). Enterococci were further identified to the species level by PCR using primers and conditions for the different enterococcal species, as described previously (Torres et al., 2003).

III.2.3.2. Antimicrobial susceptibility test

Antibiotic susceptibility was tested by the agar disk diffusion method as recommended by the CLSI (CLSI, 2010). The susceptibility of the *E. coli* isolates was tested for 16 antibiotics: [ampicillin (10 μg), amoxicillin+clavulanic acid (20 μg + 10 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), aztreonam (30 μg), imipenem (10 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), streptomycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), sulfamethoxazole-trimethoprim (SXT) (1.25 μg + 23.75 μg), tetracycline (30 μg), and chloramphenicol (30 μg)]. *E. coli* ATCC 25922 was used as a quality control strain. Additionally, screening test for detection of ESBLs was carried out by the double disk diffusion test (Bradford, 2001; CLSI, 2010).

The susceptibility of the enterococcal isolates was tested for 11 antibiotics: [vancomycin (30 μg), teicoplanin (30 μg), Ampicillin (10 μg), streptomycin (300 μg), gentamicin (120 μg), kanamycin (120 μg), chloramphenicol (30 μg), tetracycline (30 μg), erythromycin (15 μg), quinupristin-dalfopristin (15 μg), and ciprofloxacin (5 μg)] by the disk diffusion method (CLSI, 2010). Only the category of high-level resistance was considered for streptomycin (HLR-S), gentamicin (HLR-G) and kanamycin (HLR-K). *Enterococcus faecalis* strain ATCC 29212 and *Staphylococcus aureus* strain ATCC 25923 were used as quality controls.

III.2.3.3. Antibiotic resistance genes

The presence of genes encoding TEM and SHV β-lactamases was studied by PCR in all ampicillin-resistant isolates, using primers and conditions reported previously (Briñas et al., 2002). The following genes were studied by PCR: *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(E) (in tetracycline-resistant isolates), *aad*A1 (in streptomycin-resistant isolates), *aac*(3)-II and *aac*(3)-IV (in gentamicin-resistant isolates), *aac*(6´)-Ib (in amikacin-resistant isolates), *cml*A (in chloramphenicol-resistant isolates), and *sul*1, *sul*2 and *sul*3 (in SXT-resistant isolates). The presence of the *intI*1 and *intI*2 genes, encoding class 1 and 2 integrases, respectively (Radhouani et al., 2009), and genes encoding different virulence factors (*fim*A, *pap*GIII, *stx*, *cnf*1, *pap*C and *aer*) was also verified by PCR using primers and conditions previously described (Ruiz et al., 2002).

Resistant enterococci isolates were tested by PCR for detection of the following resistance genes: *erm*(B) (in erythromycin resistant isolates), *tet*(M) and *tet*(L) (in tetracycline-resistant isolates), *aph*(3′)-IIIa (in kanamycin-resistant isolates), *aac*(6′)-*aph*(2″) (in gentamicin-resistant isolates), *ant*(6)-Ia (in streptomycin-resistant isolates), *vat*(D) and *vat*(E) (in quinupristin/dalfopristin-resistant isolates), using primers and conditions previously reported (Aarestrup et al., 2000; Leener et al., 2005; Torres et al., 2003). Specific PCR assays for detection of resolvase gene *tdn*X and *int* genes were also used in *tet*(M)-positive isolates, to demonstrate the presence of the Tn*5397*-like and Tn*916*/Tn*1545*-like transposons, respectively (Agerso et al., 2006).

Positive and negative controls were used in all PCRs, from the strain collection of the University of Trás-os-Montes and Alto Douro (Portugal). DNA sequencing was used to verify the identity of the gene products of at least one isolate randomly selected for each gene.

III.2.3.4. Detection of phylogenetic groups

The *E. coli* isolates were assigned to one of the four main phylogenetic groups, A, B1, B2 and D, following a PCR strategy published previously based on the presence or absence of *chu*A, *yja*A genes or the DNA fragment TSPE4.C2 (Clermont et al., 2000).

III.2.4. Results

III.2.4.1. Bacteria isolation

Thirty-six *E. coli* isolates were recovered from the 42 common buzzard faecal samples (85.7%), whilst enterococci were detected in 31 (73.8%) of the faecal samples studied. *E. faecium* was the most prevalent detected species in common buzzards (48.4%), followed by *E. faecalis* (16.1%), *E. hirae* and *E. durans* (12.9%, respectively). Three enterococci isolates could not be identified to the species level and they are referred as *Enterococcus* species.

III.2.4.2. Antimicrobial resistance among E. coli isolates

Table III.2.1 shows the percentages of the 36 *E. coli* isolates that were resistant to each of the antimicrobials tested. The double disk synergy test for detection of ESBLs was negative for all our *E. coli* isolates.

A high percentage of resistance to streptomycin and tetracycline was observed in our *E. coli* isolates (75%). More than 60% of the isolates were resistant to ampicillin.

The percentages of *E. coli* isolates that were resistant to ciprofloxacin, amikacin, cefoxitin, tobramycin or chloramphenicol ranged from 50% to 41.7%. Amoxicillin/clavulanic acid, nalidixic acid, SXT and gentamicin resistances were also present in *E. coli* isolates (38.9, 33.3, 22.2, 19.4%, respectively). Lower percentages of resistance were observed to imipinem and aztreonam (5.5 and 2.8%, respectively). All the isolates were susceptible to ceftazidime and cefotaxime. It is interesting to underline the high diversity of the resistance phenotypes; in fact, 34 different phenotypic profiles were observed in our *E. coli* isolates.

The presence of antibiotic resistance genes was studied by PCR in all resistant *E. coli* isolates. The presence of β-lactamase genes was investigated in all 22 ampicillin-resistant isolates, with the bla_{TEM} gene detected in 20 of them and the bla_{SHV} gene not being detected. The *aac*(3)-II or *aac*(3)-IV genes, encoding an aminoglycoside acetyltransferase that modifies gentamicin, was found in the seven gentamicin-resistant isolates. In addition, the *aad*A1 gene, encoding an aminoglycoside adenyltransferase that modifies streptomycin, was detected in eight of the 27 streptomycin-resistant isolates of this study. The *tet*(A) and/or *tet*(B) genes, associated with an active efflux system, were identified in 16 of the 27 tetracycline-resistant isolates. The *cml*A gene was found in three of the 15 chloramphenicol-resistant isolates. A total of eight *E. coli* isolates presented the SXT-resistant phenotype and the *sul*1 and/or *sul*2 and/or *sul*3 genes were detected in all of them. The *intI*1 gene encoding class 1 integrase and the *intI*2 gene encoding class 2 were found in four and one of the eight SXT-resistant isolates, respectively.

Table III.2.1 | Distribution of antibiotic resistance in *E. coli* and *Enterococcus* spp. isolated from faecal samples of common buzzards

Antimicrobial agent	No. (%) of *E. coli* isolates (n=36)	No. (%) of enterococci isolates distributed by species				
		E. faecium (n=15)	*E. faecalis* (n=5)	*E. durans* (n=4)	*E. hirae* (n=4)	*Enterococcus* spp. (n=3)
Ampicillin	22 (61.1)	3 (20)	0	0	0	0
Amoxicillin/ clavulanic acid	14 (38.9)	NT	NT	NT	NT	NT
Cefoxitin	16 (44.4)	NT	NT	NT	NT	NT
Cefotaxime	0	NT	NT	NT	NT	NT
Ceftazidime	0	NT	NT	NT	NT	NT
Aztreonam	1 (2.8)	NT	NT	NT	NT	NT
Imipenem	2 (5.5)	NT	NT	NT	NT	NT
Gentamicin	7 (19.4)	3 (20)	0	0	1	0
Amikacin	17 (47.2)	NT	NT	NT	NT	NT
Tobramycin	15 (41.7)	NT	NT	NT	NT	NT
Streptomycin	27 (75)	4 (26.7)	1	3	2	1
Nalidixic acid	12 (33.3)	NT	NT	NT	NT	NT
Ciprofloxacin	18 (50)	5 (33.3)	2	1	0	1
Sulfamethoxazole/ trimethoprim	8 (22.2)	NT	NT	NT	NT	NT
Tetracycline	27 (75)	14 (93.3)	4	4	3	2
Chloramphenicol	15 (41.7)	3 (20)	1	2	2	1
Vancomycin	NT	0	0	0	0	0
Teicoplanin	NT	0	0	0	0	0
Kanamycin	NT	4 (26.7)	2	2	1	0
Erythromycin	NT	13 (86.7)	3	4	3	2
Quinupristin/ dalfopristin*	NT	8 (53.3)	-	3	4	2
Susceptible to all antibiotics	1 (2.8)	0	0	0	0	0

NT, Not tested
*Susceptibility for this drug combination was not tested in *Enterococcus faecalis* isolates

III.2.4.3. Phylogenetic groups and virulence factor genes among E. coli isolates

Table III.2.2 presents the phylogenetic groups of the 36 *E. coli* isolates in relation to the virulence factors genes. Most of the isolates belonged to phylogenetic groups A (17 isolates) or D (11 isolates), and only five isolates corresponded to B2 group, and three isolates to B1 group. All the *E. coli* isolates from B1 and B2 groups carried the *fim*A gene. This virulence factor gene was also detected in almost 53 and 46% of our *E. coli* isolates, respectively. It is interesting to point out that almost all the isolates that carried the *aer* and *pap*C genes belonged to B2 and D phylogenetic groups.

Table III.2.2 | Virulence factor genes and phylogenetic groups detected among 36 *E. coli* isolates recovered from common buzzards

Virulence factor gene detected	No. (%) of *E. coli* isolates distributed by phylogenetic group			
	A (n=17)	B1 (n=3)	B2 (n=5)	D (n=11)
*fim*A	9 (52.9)	3	5	5 (45.5)
aer	0	0	2	2 (18.2)
*pap*C	0	1	1	3 (27.3)

III.2.4.4. Antimicrobial resistance among enterococci isolates

Table III.2.1 shows the percentage of antimicrobial resistance according to enterococcal species. All 31 enterococci isolates were resistant to one or more than one antibiotic agents. A higher level of resistance was observed for tetracycline (87.1%) and erythromycin (80.6%), with moderate percentage of resistance to quinupristin/dalfopristin (65.4%) and streptomycin (35.5%). Almost 30% of our isolates present resistances to ciprofloxacin, kanamycin and chloramphenicol. Low percentages were observed for ampicillin (20%) and gentamicin (12.9%). Three ampicillin-resistant *E. faecium* isolates were detected in our report. No teicoplanin- or vancomycin-resistant enterococci were identified in this study.

From the 27 tetracycline-resistant enterococcal isolates, the *tet*(M) (eight isolates), *tet*(L) (five isolates) and *tet*(M)+*tet*(L) (seven isolates) were detected.

Genes specific for Tn*916*/Tn*154* transposons were detected in eight (two *E. faecium*, two *E. faecalis*, three *E. durans* and onr *E. hirae*) of the 15 *tet*(M)-positive isolates, and specific sequences of Tn*5397* were also identified in one *E. durans* isolate. The presence of *erm*(B) gene was investigated in the 25 erythromycin-resistant isolates and was found in 80% of those isolates (ten *Enterococcus faecium*, two *Enterococcus faecalis*, three *Enterococcus durans*, three *Enterococcus hirae*, and two *Enterococcus* species isolates). The streptogramin A resistance genes *vat*(D) and/or *vat*(E) were found in nine of the 17 quinupristin-dalfopristin-resistant isolates (five *Enterococcus faecium*, two *Enterococcus durans* and two *Enterococcus hirae*). The *cat*A gene was present in one of chloramphenicol-resistant isolate (*Enterococcus durans*) In addition, the enterococcal isolates showing high-level resistance to gentamicin and streptomycin contained the *aac*(6′)-*aph*(2″) and *ant*(6)-Ia genes, respectively. Furthermore, the *aph*(3′)-IIIa gene was found in all the isolates with high-level resistance to kanamycin (four *Enterococcus faecium*, two *Enterococcus faecalis*, two *Enterococcus durans* and one *Enterococcus hirae*).

III.2.5. Discussion

Few data do exist about the susceptibility to antimicrobial agents in *E. coli* and enterococal isolates of healthy wild animals (Guenther et al., 2010; Silva et al., 2010), and even less few in common buzzards. Usually, the reports are restricted to the analysis of ESBL-containing *E. coli* and

vancomycin-resistant enterococci (Literak et al., 2010; Pinto et al., 2010; Poeta et al., 2009; Radhouani et al., 2010a; Radhouani et al., 2009).

In our study, it is important to point out that, of the 36 *E. coli* isolates, 35 of them present resistance to one or more than one antibiotic. The most prevalent resistances were to streptomycin, tetracycline and ampicillin. High rates of resistance to the same antibiotics were detected in *E. coli* isolated from migratory Canadian geese (Middleton & Ambrose, 2005), comparable to our results, whilst antimicrobial susceptibility data for wild birds have been restricted to Black headed Gulls from Czech Republic (Dolejska et al., 2007), yellow-legged Gulls from Portugal (Radhouani et al., 2009) and European wild birds of different species (Guenther et al., 2010). Interestingly the data indicate that both the synanthropic species, such as pigeons or gulls, which have frequent contact to humans, and bird species living in more rural areas and birds of prey seem to play a role as carriers of multiresistant isolates.

Almost all ampicillin-resistant *E. coli* isolates from buzzards harbored the bla_{TEM} gene. The TEM β-lactamase is the most often mechanism of ampicillin resistance in *E. coli* from different origins (Briñas et al., 2002). In our report, all the gentamicin-resistant isolates carried the *aac*(3)-II and/or *aac*(3)-IV genes. These genes, which present cross-resistance to other aminoglycosides, can be mobilized on multi-resistance elements, so that the spread of gentamicin-resistant determinants is likely to be selected by antimicrobial agents other than gentamicin (Jakobsen et al., 2007). The detection of *tet*(A) and/or *tet*(B) genes in almost 60% of our tetracycline-resistant isolates shows that the main mechanism of tetracycline resistance in *E. coli* isolates from common buzzards is by active efflux.

All our SXT-resistant *E. coli* isolates carried *sul* genes. This high occurrence is similar to that reported in previous studies (Soufi et al., 2009; Vinue et al., 2010). Four of the eight SXT-resistant isolates possessed the *sul*3 gene, high-lighting the high ability of this gene to disseminate in different populations, possibly due to the efficient genetic structure in which this gene is included.

In our study, three of the eight SXT-resistant isolates carried class 1 integrons, and one of them contained both class 1 and class 2 integrons. The high prevalence of integrons is a cause of concern, mainly due to the significant association of integrons with ampicillin resistance and even with multi-resistance phenotypes. The presence of integrons among commensal *E. coli* from common buzzards is a cause for alarm because this genetic structure is very efficient for the acquisition of antimicrobial resistance genes, which could be transmitted to other bacteria by mobile elements such as plasmids and transposons. Integrons appear to occur not only among clinical isolates of *E. coli* but also among commensal strains, even in wild animals.

It is well recognized that *E. coli* consists of a number of distinct phylogroups and that isolates of the different phylogroups differ in their ecological niches, life-history characteristics and propensity to be the origin of diseases. Consequently, much can be learnt by assigning a strain of *E. coli* to one of the recognized phylogroups (Gordon et al., 2008). In our study, phylogenetic typing revealed an affiliation of a high proportion of multiresistant strains to groups A and D. The same

results have been obtained in poultry meat (Soufi et al., 2009) and in wild animals (Poeta et al., 2007a; Radhouani et al., 2010a; Radhouani et al., 2009). At least one virulence-associated gene (*fim*A, *pap*C or *aer*) was detected in 22 of the 36 studied isolates (61%). The same was observed in *E. coli* isolates from poultry meat (Soufi et al., 2009). The emergence of potentially highly virulent isolates in combination with a multi-resistance phenotype is alarming, as a possible consequence would be a severe clinical outcome concomitant with serious limitations in antimicrobial treatment.

Among our enterococcal isolates, *Enterococcus faecium* and *Enterococcus faecalis* were the most predominant enterococcal species in the faecal samples of common buzzards. This observation is in agreement with those of other studies performed in animals (Aarestrup et al., 2000; Kojima et al., 2010) and particularly in wild animals in Portugal (Poeta et al., 2007a; Silva et al., 2010).

It is important to highlight the presence of three ampicillin-resistant *Enterococcus faecium* isolates. Recently, it has been relatively common to find *Enterococcus faecium* resistant to this antibiotic, as a result of modifications in its penicillin-binding proteins (PBP5), although this resistance phenotype has been identified more in isolates of human origin (Billstrom et al., 2008). Our results are consistent with the results of others studies in humans, poultry and pets (Poeta et al., 2005a) and in wild animals (Poeta et al., 2007a; Silva et al., 2010).

In our report, the *erm*(B), *tet*(M) and/or *tet*(L) genes identified were frequently associated together in the same strain. The *erm*(B) gene is frequently linked with the *tet*(M) gene on the highly mobile conjugative transposon Tn*1545*, which predominates in clinically important Gram-positive bacteria (De Leener et al., 2004). It has been suggested that there may be a correlation between the level of antimicrobial resistance in faecal bacteria from animals and the level of contact of these animals with people (Radhouani et al., 2010b).

The common buzzards included in this study were localized in different natural areas in the north and the center of Portugal, were they make their nests. They are a large predator birds that are at the top of the food chain. This fact could be one answer to the high rates of antimicrobial resistance found in *E. coli* and enterococci isolates from these birds. The data presented here suggest that wild birds are common carriers of multiresistant faecal bacteria, and are thus probably involved in the transmission of antimicrobial resistance into the environment. In particular, common buzzards seem to represent an important reservoir, or at least source, of multiresistant *E. coli* and enterococci isolates, and consequently may represent a considerable hazard to human and animal health by transmission of these isolates to waterways and other environmental sources via their faecal deposits. Most obviously, *E. coli* and enterococci of common buzzards seem to reveal the same resistance patterns as isolates isolated from other animals, thus highlighting the need for thorough future epidemiological studies to gain a more detailed understanding of the transmission mode of resistant bacteria to wild birds and back into the environment.

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III.3. Antimicrobial resistance and virulence genes in *Escherichia coli* and enterococci from red foxes (*Vulpes vulpes*)

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III.3.1. Abstract

The aims of the study were to analyse the prevalence of antimicrobial resistance and the mechanisms implicated, as well as the virulence factors, in faecal *Escherichia coli* and *Enterococcus* spp. from red foxes. From 52 faecal samples, 22 *E. coli* (42.3%) and 50 enterococci (96.2%) isolates were recovered (one/sample). A high percentage of *E. coli* isolates exhibited resistance to streptomycin, tetracycline, trimethoprim-sulfamethoxazole or ampicillin (54-27%), and they harboured the *aad*A, *tet*(A) and/or *tet*(B), *sul*1 and bla_{TEM} resistance genes, respectively. The *E. coli* isolates were ascribed to the 4 major phylogroups, D (41% of isolates), A (31.8%), B1 (18.2%) and B2 (9.1%), and carried the *fim*A (63.3%) or *aer* (13.6%) virulence genes. Among enterococcal isolates, *E. faecium* was the most prevalent species (50%). A high percentage of enterococcal isolates showed tetracycline resistance (88%) harbouring different combinations of *tet*(M) and *tet*(L) genes. The *erm*(B) or the *aph*(3′)-IIIa gene were identified in most of our erythromycin- or kanamycin-resistant enterococci, respectively. This report suggests the role of red foxes from rural areas in the cycle of transmission and spread of antimicrobial resistant *E. coli* and enterococci into the environment, representing a reservoir of these antimicrobial-resistant microorganisms.

III.3.2. Introduction

The emergence and on-going spread of antimicrobial-resistant bacteria is a major public health threat. Antimicrobial drugs have been widely used in human, veterinary and agricultural practices providing the selective pressure for emergence and persistence of acquired resistance determinants. Though, resistance has also been observed in the absence of antimicrobial exposure, such as in bacteria from wildlife, raising a question about the mechanisms of emergence and persistence of antimicrobial-resistant strains (Thaller et al., 2010).

The bacteria of the normal microbiota of the gut, such as *Escherichia coli* and enterococci, can easily acquire and transfer resistance genes (Sorum & Sunde, 2001). These commensal bacteria, that constitute a reservoir of resistance genes for pathogenic bacteria, can thus be used as indicators of changes in antimicrobial resistance (Caprioli et al., 2000).

In contrast to reports on the appearance of antimicrobial-resistant *E. coli* and enterococci in humans and domesticated animals, their presence in wildlife, particularly in red foxes, has been addressed in few occasions. In fact, just two previous studies were performed in antimicrobial-resistant *E. coli* of red foxes, one in Germany (Grobbel et al., 2012) and the other one in Slovakia (Literak et al., 2010).

In addition, our research group has previously performed studies on red foxes, but focused on the analysis of specific mechanisms of antimicrobial resistances, as is the case of extended-spectrum beta-lactamases (ESBL) in *E. coli* (Costa et al., 2006; Radhouani et al., 2012a) or vancomycin-resistance in enterococci (Radhouani et al., 2011), using for that purpose selective antimicrobial-supplemented plates for the recovery of resistant isolates.

The aim of the present study was to analyse the prevalence of antimicrobial resistance and the mechanisms implicated, as well as the virulence factors in randomly-selected faecal *E. coli* and *Enterococcus* spp. isolates of red foxes (obtained without previous selection on antimicrobial-supplemented media).

III.3.3. Material and methods

III.3.3.1. Samples and bacteria

Fifty-two red fox (*Vulpes vulpes*) faecal samples were studied in this report. Samples were collected (one sample per individual) from February-2008 to March-2009 in North of Portugal (where these animals live in the wild) during hunts of red foxes. This kind of hunting is organized all of the years during a short period of time having like main objective the ecological control of the animal population. The animals were obtained in collaboration with National Corporation of Forest Rangers. As far as we know, none of the animals had received antimicrobials.

For *E. coli* isolation, faecal samples were seeded in Levine agar plates and incubated 24 h at 37°C and colonies with typical *E. coli* morphology were selected and identified by classical biochemical methods (Gram, catalase, oxidase, indol, methyl-red-voges-proskauer, citrate and urease), and confirmed by the API 20E system (BioMérieux, La Balme Les Grottes, France). These faecal samples were previously analysed for the presence of extended-spectrum beta-lactamases *E. coli* isolates using cefotaxime-supplemented Levine agar plates for the recovery of resistant isolates (Radhouani et al., 2012a).

For enterococcal isolation, faecal samples were sampled in Slanetz–Bartley agar plates and were incubated for 48 h at 37°C. Colonies with typical enterococcal morphology were identified by cultural characteristics, Gram-staining, catalase test, bile-aesculin reaction and by biochemical tests using the API ID20 Strep system (BioMérieux, La Balme Les Grottes, France). Furthermore, enterococci were identified to the species level by PCR (Torres et al., 2003). These faecal samples were previously analysed for the presence of vancomycin-resistant enterococci using vancomycin-supplemented Slanetz–Bartley agar plates for the recovery of resistant isolates (Radhouani et al., 2011).

III.3.3.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by the agar disk diffusion method as previously recommended (CLSI, 2010). The susceptibility of the *E. coli* isolates was tested for 16 antimicrobials: [ampicillin, amoxicillin+clavulanic acid, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, gentamicin, amikacin, tobramycin, streptomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole-trimethoprim (SXT), tetracycline, and chloramphenicol]. *E. coli* ATCC 25922 was used as a quality control strain. Additionally, screening test for detection of ESBLs was carried out by the double disk diffusion test (Bradford, 2001; CLSI, 2010).

On the other hand, the susceptibility of the enterococcal isolates was tested for 11 antimicrobials: [vancomycin, teicoplanin, ampicillin, streptomycin, gentamicin, kanamycin, chloramphenicol, tetracycline, erythromycin, quinupristin-dalfopristin, and ciprofloxacin]. Only the category of high-level resistance was considered for streptomycin (HLR-S), gentamicin (HLR-G) and kanamycin (HLR-K). *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains.

III.3.3.3. Antimicrobial-resistance genes

The following antimicrobial resistance genes were studied by PCR: bla_{TEM} and bla_{SHV} (in ampicillin-resistant isolates), *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(E) (in tetracycline-resistant isolates), *aad*A (in streptomycin-resistant isolates), *aac*(3)-II and *aac*(3)-IV (in gentamicin-resistant isolates), *aac*(6′)-Ib (in amikacin-resistant isolates), *cml*A (in chloramphenicol-resistant isolates), and *sul*1, *sul*2 and *sul*3 (in SXT-resistant isolates). The presence of the *intI*1 and *intI*2 genes, encoding respectively class 1 and 2 integrases, was also investigated (Radhouani et al., 2009).

On the other hand, resistant enterococci were tested by PCR for detection of the following resistance genes: *erm*(B) (in erythromycin resistant isolates), *tet*(M) and *tet*(L) (in tetracycline-resistant isolates), *aph*(3′)-IIIa (in kanamycin-resistant isolates), *aac*(6′)-*aph*(2″) (in gentamicin-resistant isolates), *ant*(6)-Ia (in streptomycin-resistant isolates), *cat*A (in chloramphenicol-resistant isolates), *vat*(D) and *vat*(E) (in quinupristin/dalfopristin-resistant isolates). A PCR assay was used to demonstrate the presence of the Tn*5397*-like and Tn*916*/Tn*1545*-like transposons in *tet*(M)-positive isolates (Radhouani et al., 2010b). Positive and negative controls were used in all PCRs, from the strain collection of the University of Trás-os-Montes and Alto Douro (Portugal). DNA sequencing was used to verify the identity of the gene products of at least one isolate randomly selected for each resistance gene.

III.3.3.4. Virulence factor genes

The presence of genes encoding different virulence factors for *E. coli* isolates (*fim*A, *pap*GIII, *stx*, *cnf*1, *pap*C and *aer*) and for enterococci (*ace*, *agg*, *cpd*, *gel*E, *esp*, *hyl, fsr* and *cyl*L_LL_SABM) was also analysed by PCR (Radhouani et al., 2010b).

III.3.3.5. Detection of phylogenetic groups

The detection of phylogenetic groups of *E. coli* isolates was performed by PCR (Clermont et al., 2000).

III.3.4. Results and discussion

E. coli isolates were recovered from 22 of the 52 tested faecal samples (42.3%) and enterococcal isolates from 50 of the 52 samples (96.2%). One *E. coli* or enterococcal isolate per positive sample was selected and further studied. *E. faecium* was the most prevalent enterococcal species (50%), followed by *E. faecalis* (22%), *E. hirae* (16%) and *E. durans* (2%), and species identification was not possible for some enterococci (Table III.3.1).

Sixteen of the 22 *E. coli* isolates (72.7%) showed resistance to one or more of tested antimicrobial agents. A high percentage of *E. coli* isolates exhibited resistance to streptomycin, tetracycline, SXT and ampicillin (54.4%, 50%, 31.8% and 27.3%, respectively). Nalidixic acid resistance was observed in almost 19% of *E. coli* isolates. Furthermore, a lower percentage of resistance was identified to chloramphenicol and tobramycin (4.5%). The double disk synergy test for detection of ESBLs was negative for all our *E. coli* isolates. Other reports have found that the most common antimicrobial resistances observed among *E. coli* isolates from swine, poultry and cattle were against tetracycline, ampicillin, aminoglycosides and sulfamethoxazole (Guerra et al., 2003). All our isolates showed susceptibility to amoxicillin+clavulanic acid, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, gentamicin, amikacin and ciprofloxacin.

The physiological cost of resistance in the absence of antimicrobial pressure may have consequently caused the loss of some of this genetic material (Benavides et al., 2012). This loss could explain the low prevalence of resistance found in red foxes and the differences in antimicrobial-resistant phenotypes between the isolates coming from humans or farm animals and wild animals. Further analysis of the genetic component of antimicrobial phenotypes studied here in our report could help elucidate the origin and mechanisms of antimicrobial-resistant spread among wild animals.

Table III.3.1 shows the antimicrobial-resistance genes detected among our isolates. The bla_{TEM}, *tet*(A), *tet*(B), *aad*A or *sul*1 genes were identified in our ampicillin, tetracycline, streptomycin and sulfamethoxazole resistant *E. coli* isolates, respectively. The TEM β-lactamase is the most frequent mechanism of ampicillin resistance in *E. coli* isolates from different origins (Brinas et al., 2002). The detection of *tet*(A) or *tet*(B) genes in tetracycline-resistant isolates indicates that the main mechanism of tetracycline resistance among these isolates is by active efflux (Radhouani et al., 2009). Moreover, the high occurrence of *sul*1 gene in our survey is similar to that reported in other previous studies (Soufi et al., 2009).

It is important to point out that almost 41% of the *E. coli* isolates belonged to the phylogroup D. The other isolates belonged to phylogroups A (31.8%), B1 (18.2%) or B2 (9.1%). Different reports have revealed that these phylogroups differ in the presence of virulence factors, ecological niches and life-history and some characteristics, such as their ability to exploit different sugar sources, their antimicrobial-resistance profiles and their growth rate (Carlos et al., 2010).

Various researchers analysed the distribution of the main phylogenetic groups among *E. coli* strains isolated from human and animal faeces (Carlos et al., 2010). It was found that the relative abundance of phylogenetic groups among mammals is dependent on the host diet, body mass and climate (Gordon & Cowling, 2003). Furthermore, another study observed the prevalence of groups D and B1 in birds, groups A and B1 in non-human mammals, and groups A and B2 in humans. These different reports concluded that one of the main forces shapes the genetic structure of *E. coli* populations among the hosts is domestication (Escobar-Paramo et al., 2006). In our report, phylogenetic typing revealed an affiliation of a high proportion of multiresistant strains to groups A and D. Similar results were observed in chickens and swine (Machado et al., 2008), in wild boars (Poeta et al., 2009) and in wild birds (Radhouani et al., 2010a; Radhouani et al., 2012b) in the same geographical area.

The majority of the *E. coli* isolates that carried *fim*A gene belonged to D phylogroup and the *pap*C gene was identified in three *E. coli* isolates (Table III.3.2). Several reports showed that strains belonging to phylogroups B2 and D contained more virulence factors than strains from the phylogroups A and B1 (Escobar-Paramo et al., 2006; Johnson et al., 2001).

Table III.3.1 | Resistance genes detected in antibiotic resistant *E. coli* and enterococcal isolates obtained from red fox faecal samples

Bacteria	Phenotype of resistance	Number of isolates	Genes detected by PCR	
			Resistance genes or genetic elements studied	Number of isolates
E. coli	Ampicillin	6	bla_{TEM}	3
	Streptomycin	12	*aad*A	6
	Sulfamethoxazole/trimethoprim	7	*sul*1	5
			*intI*1	5
	Tetracycline	11	*tet*(A)	7
			tet(B)	2
			tet(A) + *tet*(B)	1
	Chloramphenicol	1	*cml*A	0
E. faecium	Gentamicin	3	*aac*(6′)-*aph*(2″)	3
	Streptomycin	4	*ant*(6)-Ia	1
	Tetracycline	24	*tet*(M)	2
			tet(L)	3
			tet(M) + *tet*(L)	16
			Tn*916*	6
			Tn*916* + Tn*5397*	1
	Chloramphenicol	5	*cat*(A)	1
	Kanamycin	6	*aph*(3′)-IIIa	6
	Erythromycin	14	*erm*(B)	11
	Quinupristin/dalfopristin[a]	5	*vat*(E) and/or *vat*(D)	0
E. faecalis	Streptomycin	2	*ant*(6)-Ia	0
	Tetracycline	9	*tet*(M)	2
			tet(M) + *tet*(L)	5
			tet(L)	1
			Tn*916*	2
	Chloramphenicol	3	*cat*(A)	1
	Kanamycin	1	*aph*(3′)-IIIa	1
	Erythromycin	4	*erm*(B)	2
E. durans	Streptomycin	1	*ant*(6)-Ia	1
	Tetracycline	1	*tet*(M) + *tet*(L)	1
			Tn*916*	1
	Kanamycin	2	*aph*(3′)-IIIa	2
	Erythromycin	1	*erm*(B)	1
E. hirae	Streptomycin	1	*ant*(6)-Ia	1
	Tetracycline	6	*tet*(L)	1
			tet(M) + *tet*(L)	5
			Tn*916*	3
	Kanamycin	1	*aph*(3′)-IIIa	1
	Erythromycin	6	*erm*(B)	4
	Quinupristin/dalfopristin[a]	1	*vat*(E) and/or *vat*(D)	0
Enterococcus spp	Streptomycin	2	*ant*(6)-Ia	0
	Tetracycline	4	*tet*(L)	1
			tet(M)	2
			tet(M) + *tet*(L)	1
			Tn*916*	1
	Chloramphenicol	1	*cat*(A)	0
	Kanamycin	2	*aph*(3′)-IIIa	2
	Erythromycin	2	*erm*(B)	0
	Quinupristin/dalfopristin[a]	3	*vat*(E) and/or *vat*(D)	0

[a] Susceptibility for this drug combination was not tested in *Enterococcus faecalis* isolates

Table III.3.2 | Virulence genes and phylogenetic groups detected among 22 *E. coli* isolates recovered from red fox faecal samples

Virulence gene detected	Number of *E. coli* isolates distributed by phylogenetic groups			
	A (n=7)	B1 (n=4)	B2 (n=2)	D (n=9)
*fim*A	3	3	2	6
*pap*C	1	1	0	1
None virulence genes	4	0	0	2

Phenotypic profiles of enterococci isolates based on the susceptibility to multiple antimicrobials that are used in our study provided some important observations. Most evident is the magnitude of resistance to individual classes of antimicrobials across all isolated enterococci, with 88% resistant to tetracycline, 54% to erythromycin, 40% to quinupristin-dalfopristin, 18% to chloramphenicol, 14% to ciprofloxacin, 12% to ampicillin, and 24%, 20% and 6% resistant to the aminoglycosides kanamycin, streptomycin and gentamicin, respectively.

In our report, the most prevalent resistance phenotypes in wildlife *E. coli* and enterococci are resistance to streptomycin-tetracycline and tetracycline-erythromycin, respectively. These patterns are very similar to the patterns for isolates from other wild animals as wild rabbits (Silva et al., 2010) and wild birds (Guenther et al., 2010b; Radhouani et al., 2012b); and also very common in human and livestock populations in Europe (Guenther et al., 2011). Antimicrobial-resistant bacteria have been commonly isolated from a variety of sources, including domestic sewage, drinking water, rivers, and lakes (Sayah et al., 2005). Antimicrobials from both urban and agricultural sources persist in soil and aquatic environments, and the selective pressure imposed by these compounds may affect the treatment of human diseases. The selective pressure applied by the antimicrobials that are used in clinical and agricultural settings has promoted the evolution and spread of genes that confer resistance, regardless of their origins (Allen et al., 2010). In addition to the consequences for human health, interests and preoccupations have been increased about the contamination of surface water with resistant bacteria from livestock operations and human garbage.

In our study, the enterococci isolates presented diverse resistance gene profiles, the most prevalent genotype included *tet*(M)+*tet*(L) and *erm*(B) genes (Table III.3.1). In addition, the *aac(*6′)-*aph*(2″), *ant*(6)-Ia and *aph*(3′)-IIIa genes detected in our high-level aminoglycoside-resistant enterococci isolates, were also found in a previous report among HLR-G, HLR-S and HLR-K enterococci from swine (Jackson et al., 2005); and in wild animals (Radhouani et al., 2010b).

Furthermore, the presence of various virulence factors has been indicated for the widespread presence of the enterococci. The virulence factors such as aggregation substance (*agg*), cytolysin (*cyl*), hyaluronidase (*hyl*), the enterococcal surface protein (*esp*), the pheromone determinant (*cpd*) and gelatinase (*gel*E) are often reported (Jahangiri et al., 2010). Several studies revealed that these virulence factors are possibly associated with the colonisation and pathogenesis of enterococci (Jett

et al., 1992). The *cpd*, *gel*E, *hyl*, *esp*, *agg*, *cyl*L_L, *cyl*L_S and *cyl*M genes were detected in 24%, 22%, 20%, 18%, 16%, 14%, 6% and 4% of our enterococci, respectively. Thirty-one of 50 enterococcal isolates (19.4%) carried at least one virulence factor gene. Of *E. faecium* isolates, 60% were positive for one or more virulence determinants, compared to 72.7% of *E. faecalis* isolates. Among the diverse virulence profiles, *gel*E and *esp* profile was the most common among our enterococci (6 *E. faecium*). Certain focus has been placed on the large surface protein Esp (enterococcal surface protein) and on the activity of gelatinase (Marra et al., 2007; Sedgley et al., 2005). Reports of genes contained in clinical and environmental isolates have implicated those for Esp and gelatinase because of their association with infections at particular sites (Mohamed & Murray, 2005; Semedo et al., 2003). In fact, these proteins are of interest for their roles in biofilm formation in vitro (Marra et al., 2007).

III.3.5. Conclusion

This study suggests that there is a reservoir of antimicrobial-resistant *E. coli* and enterococci among red foxes (*Vulpes vulpes*). Foxes can go into human habitat and the incidence of antimicrobial-resistant *E. coli* and enterococci in red foxes may be due to their diet as these wild animals frequently hunt poultry, rabbits, and birds and also scavenge human garbage; in this sense, can easily be in contact with food that could be contaminated by resistant isolates of human and animal origin (Literak et al., 2010). These wild animals can easily pickup human waste and usually interact with human faeces in the sewage system in urban environments and can therefore easily acquire multiresistant-bacteria. Extraordinarily, red foxes have also been described as hosts of these bacteria, which might expose their omnivorous feeding behaviour. They are on top of the food chain, perhaps accumulating multiresistant bacteria from their prey. Furthermore, several reports revealed that the antimicrobial-resistance genes found in red foxes are also found in their prey (Guenther et al., 2010a; Guenther et al., 2010b; Silva et al., 2010).

Our report showed that areas with high livestock and human density and an assumable frequent interaction of wildlife with human influenced habitats of any kind (livestock farms, landfills, sewage systems, or wastewater treatment facilities) result in a higher risk for wildlife to acquire antimicrobial-resistant bacteria. Moreover, the eventual proximity of large cities can represent a hazard, since some foxes may colonise urban areas. The red fox is active during all seasons, and most of its activity takes place at night or at twilight, but occasionally during daylight. When in residential areas, even at night, the foxes used corridors of vegetation wherever possible, moved quickly across open areas such as roads, and appeared to limit their foraging to patches of remnant forest, scrub, or tall grass. In this sense, fox movements may influence both the probability to get infected with resistant bacteria and the subsequent spread of these bacteria.

These microorganisms may represent a possible source of antimicrobial resistance genes, which might pose a public health hazard. Moreover, due to their predatory nature, red foxes could serve as main indicators of environmental contamination with antimicrobial-resistant bacteria. Future researches should report whether antimicrobial-resistance genes could persist in the environment or circulate in animals for long periods and may thus be disseminated by wildlife and other vectors. Thorough temporal and spatial studies of antimicrobial drug resistance in different natural habitats are needed to completely understand the importance of wildlife as a source of antimicrobial resistance.

III.3.6. Acknowledgements

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III.4. Molecular characterization of antibiotic resistance in enterococci recovered from seagulls (*Larus cachinnans*) representing an environmental health problem

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III.4.1. Abstract

Antimicrobial resistance and the mechanisms implicated were studied in 54 enterococci recovered from 57 seagull faecal samples. Almost 78% of the recovered enterococci showed resistance against one or more antibiotics and these isolates were identified to the species level. *E. faecium* was the most prevalent species (52.4%). High percentages of erythromycin and tetracycline resistances were found among our isolates (95.2%), and lower percentages were identified to other antibiotics. Most of tetracycline-resistant strains carried the *tet*(M) and/or *tet*(L) genes. Genes associated with Tn*916*/Tn*1545* and/or Tn*5397* transposons were detected in 45% percent of tetracycline-resistant isolates. The *erm*(B) gene was detected in 65% of erythromycin-resistant isolates. The *vat*(D) and *vat*(E) genes were present in 5.9% and 11.8% of quinupristin/dalfopristin-resistant isolates, respectively. The *ant*(6)-Ia gene was identified in 57.1% of streptomycin-resistant isolates. All nine kanamycin-resistant isolates carried the *aph*(3)´-IIIa gene. The *cat*A gene was found in two chloramphenicol-resistant isolates. Seagulls should be considered a risk species for spreading in the environment antimicrobial resistant enterococci and can serve as a sentinel for antibiotic pressure from the surrounding farm and urban setting.

Environmental impact: Migrating birds that fly and travel long distances can provide an opportunity to monitor the impact of antibiotic use on the natural environment and can serve as a sentinel for antibiotic pressure from the surrounding farm and urban environments. The significant presence of antibiotic resistance genes in gulls will allow tracking of the flow of resistance genes and the identification of the origins of these determinants in the environment. The study suggests that seagulls are an important host reservoir of resistant enterococci strains, probably reflecting the presence of such strains in their sources of food and/or water. Although gulls do not naturally come into contact with antibiotics, these birds can be infected with resistant enterococci and potentially serve as their reservoirs, vectors and bioindicators in the environment.

III.4.2. Introduction

Enterococci are Gram-positive and opportunistic bacteria that inhabit the gastrointestinal tract of humans and many animals. These bacteria host a wide variety of mobile genetic elements and are considered a reservoir for acquisition and distribution of antibiotic resistance genes among Gram-positive bacteria (Courvalin, 2006). In recent years, with the emergence of multidrug resistant isolates, enterococci have become an increasingly significant cause of nosocomial infections particularly caused by *Enterococcus faecalis* and *E. faecium* which are among the top types of organisms isolated worldwide (Billstrom et al., 2008).

There is a great concern about antimicrobial resistance in bacteria of human and animal origin, in particular in those that may be pathogenic, including the possibility of transfer of resistance genes from animal to human bacteria (DuPont & Steele, 1987; Phillips et al., 2004). The reported increase in antimicrobial resistance, with impact in human health, were thought to result in part as consequence of antimicrobial use for growth promotion in animals in the past (Kelly et al., 2004; Smith et al., 2003). In fact, this is due to the possibility of transferring, through the food chain, of antimicrobial-resistant strains belonging to the commensal microflora of animals to humans. Consequent development of cross-resistance to therapeutic antimicrobial agents used in the treatment of human infections is thought to arise when commensal bacteria transfer their resistant genes to others, particularly the pathogenic ones (Huycke et al., 1998). For this reason, the use of all antibiotics as growth promoters in animals was forbidden in the European Union in 2006 (EC, 2003; EC, 2008).

The Berlengas archipelago is a group of very small islands near to the Portuguese coast that are one of the first protected areas in the world. A natural heritage of great value, the small Berlengas archipelago, situated at about 10 km distance of Peniche coast, is constituted by the Berlengas Grande Island and the adjacent reefs, classified as a Natural Reserve since 1981. Presented in an almost pure and wild state, the archipelago presents an important and rich habitat for many animal and plant species, local fauna consisting mostly of sea birds. The Berlengas Natural Reserve is noted for its beautiful landscapes, with crystalline waters and charming natural nooks. Currently, the archipelago has no permanent residents and is only visited by scientists and, in the summer, by a small number of tourists. There are only a dozen of old houses that were inhabited by former fishermen and now belonged to their descendants. In the archipelago, there are no agricultural activities or livestock animals. The visitors are instructed to maintain the natural environment and preserve all the species that naturally inhabit these islands. Makeshift paths are marked with stones and guardians watch out for visitors straying into the prohibited areas, so as not to disturb the birds and the wildlife of this reserve. By these measures, the seagulls are not directly under antibiotic selective pressure in the Berlengas (Morais et al., 1995). The yellow-legged gull *Larus Cachinnans* represents the largest part of the seabirds probably due to its opportunistic feeding habits and to its few specific requirements for successful breeding. That wild bird has undergone a widespread

demographic increase for the past 40 years in these islands (Yésou & Beaubrun, 1995). Around the world, exist just a few studies of antimicrobial resistance of intestinal enterococci in wild animals (Laukova, 1999; Mallon et al., 2002; Poeta et al., 2007; Poeta et al., 2005a; Poeta et al., 2005b).

In a study performed in the past by our group, it was shown that vancomycin-resistant enterococci frequently occur in the intestinal microbiota of these birds (Radhouani et al., 2010), representing a problem in public human health. These animals are often opportunistic marine feeders, seeking their food (fish, bivalves, mollusks, eggs, birds, etc) along the shoreline or offshore, but also readily utilizing the food sources provided by humans, especially garbage. Migrating birds that fly and travel long distance seem to act as transporters, or as reservoirs, of resistant bacteria and may consequently have an important epidemiological role in the dissemination of resistance, as well as being mirrors of the spectrum of pathogenic microorganisms present in humans. If antibiotic resistance further depletes the number of effective drugs available, sick individuals will have even fewer treatment options, which represent a serious public health problem.

It is important to monitor the occurrence of different enterococcal species as well as their antimicrobial resistance profiles and resistance genes in intestinal bacteria of different animals, in order to expand the knowledge of the possible exchange of strains and their antimicrobial resistance genes among bacteria colonizing both animals and humans.

The aim of this study was to determine the occurrence of different enterococcal species in faecal samples of seagulls of Berlengas natural reserve in Portugal as well as to determine the phenotypes of antibiotic resistance of these enterococci and the implicated mechanisms of resistance. This study will give important information to know the distribution of resistance genes in different ecosystems and to understand the complex flow of these genes.

III.4.3. Materials and methods

III.4.3.1. Samples and bacteria

Fifty-seven fresh faecal droppings were obtained from seagulls (*Larus cachinnans*) in different areas of Berlengas Grande Island on five days during September 2007. To carrying out the faecal sampling, our research group needed the support of four marine biologists who agreed to help in the investigation. Four groups of two persons each one, were dispersed in different locations on the island and only the fresh faecal samples deposited by birds were collected. Our goal was to travel across the entire island to minimize the risk of collecting faecal samples of the same birds. Each sample was collected from the soil, transferred into a sterile plastic bag maintained at 4°C, and transported to the laboratory no longer than 24 hours after collection. Each sample was diluted in sterile 0.9 per cent saline solution, inoculated into Brain Heart Infusion (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated for 24 hours at 37°C. The samples were diluted and sampled in

Slanetz-Bartley agar plates (Oxoid Ltd, Basingstoke, Hampshire, England) and incubated 48 h at 35ºC. One colony with typical enterococci morphology was selected and identified from each positive sample. Colonies with typical enterococcal morphology were identified to the genus level by cultural characteristics, Gram's strain, catalase test and bile-aesculin reaction (BioMérieux, La Palme, France). Species identification was carried by PCR using primers and conditions for the different enterococcal species (Torres et al., 2003).

III.4.3.2. Antimicrobial susceptibility testing

Antibiotic susceptibility was tested for 11 antibiotics of interest in animal and human medicine (μg/disk): vancomycin (30), teicoplanin (30), ampicillin (10), streptomycin (300), gentamicin (120), kanamycin (120), chloramphenicol (30), tetracycline (30), erythromycin (15), quinupristin-dalfopristin (15), and ciprofloxacin, (5), by the disk diffusion method (C.L.S.I, 2008). Antibiotic disks were obtained from Oxoid (Oxoid Ltd, Basingstoke, UK), with the exception of aminoglycoside disks that were prepared in the laboratory. *E. faecalis* strain ATCC 29212 and *Staphylococcus aureus* strain ATCC 25923 were used for quality control.

III.4.3.3. Antibiotic resistance genes

Macrolide [*erm*(A), *erm*(B), *erm*(C)], streptogramine [*vat*(D), and *vat*(E)], tetracycline [*tet*(M), *tet*(L)], aminoglycoside [*aph*(3´)-IIIa, *aac*(6')-*aph*(2''), *ant*(6)-Ia)] and chloramphenicol [*cat*A] resistance genes were tested by PCR (Table III.4.1) in all enterococcal isolates which showed resistance for these antibiotics using primers and conditions previously reported (De Leener et al., 2005; Torres et al., 2003). Positive and negative controls, used in all PCR assays, belong to the strain collection of the University of Trás-os-Montes and Alto Douro in Portugal. A PCR assay (Table III.4.1) was used to demonstrate the presence of the Tn*5397*-like and Tn*916*/Tn*1545*-like transposons (Agersø et al., 2006; De Leener et al., 2004).

III.4.3.4. Statistical Analysis

Associations among the resistance profiles obtained for each isolate were determined using methods for clustering analysis. The results for each antimicrobial drug were coded in binary data (0 for susceptible and intermediate phenotypes and 1 for resistant ones), and the corresponding matrix was computed using NTSYS-pc software, applying the Simple Matching coefficient and UPGMA (unweighted pair group method with arithmetic average) for clustering and dendrogram construction (Rohlf, 2000).

Table III.4.1 | Target genes and primers used in the PCR reactions carried out in this study[a]

Target gene	Primer Name	Sequence (5´-3')	PCR product size (bp)
ddl *E. faecalis*	*ddl* *E. faecalis*-F *ddl* *E. faecalis*-R	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941
ddl *E. faecium*	*ddl* *E. faecium*-F *ddl* *E. faecium*-R	GCAAGGCTTCTTAGAGA CATCGTGTAAGCTAACTTC	550
E. hirae (*mur*G)	*E. hirae* (*mur*G)-F *E. hirae* (*mur*G)-R	GGCATATTTATCCAGCACTAG CTCTGGATCAAGTCCATAAGTGG	521
E. durans (*mur*2)	*E. durans* (*mur*2)-F *E. durans* (*mur*2)-R	CGTCAGTACCCTTCTTTTGCAGAGTC GCATTATTACCAGTGTTAGTGGTTG	130
erm(A)	*erm*(A)-F *erm*(A)-R	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	645
erm(B)	*erm*(B)-F *erm*(B)-R	GAAAAGRTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639
erm(C)	*erm*(C)-F *erm*(C)-R	TCAAAACATAATATAGATAAA GCTAATATTGTTTAAATCGTCAAT	642
vat(D)	*vat*(D)-F *vat*(D)-R	CCGAATCCTATGAAAATGTATCC GCAGCTACTATTGCACCATCCC	413
vat(E)	*vat*(E)-F *vat*(E)-R	ACGTTACCCATCACTATG GCTCCGATAATGGCACCGAC	282
tet(M)	*tet*(M)-F *tet*(M)-R	GTTAAATAGTGTTCTTGGAG CTAAGATATGGCTCTAACAA	576
tet(L)	*tet*(L)-F *tet*(L)-R	CATTTGGTCTTATTGGATCG CAATATCACCAGAGCAGGCT	456
aph(3')-IIIa	*aph*(3')-IIIa-F *aph*(3')-IIIa-R	GCCGATGTGGATTGCGAAAA GCTTGATCCCCAGTAAGTCA	292
aac(6')-Ie-*aph*(2'')-Ia	*aac*(6')-Ie-*aph*(2'')-Ia-F *aac*(6')-Ie-*aph*(2'')-Ia-R	CCAAGAGCAATAAGGGCATA CACTATCATACCACTACCG	220
ant(6)-Ia	*ant*(6)-Ia-F *ant*(6)-Ia-R	ACTGGCTTAATCAATTTGGG GCCTTTCCGCCACCTCACCG	577
*cat*A	*cat*A-F *cat*A-R	GGATATGAAATTTATCCCTC CAATCATCTACCCTATGAAT	486
Tn*5397*	Tn*5397*-F Tn*5397*-R	ATGATGGGTTGGACAAAGA CTTTGCTCGATAGGCTCTA	1500
Tn*916*/Tn*1545*	Tn*916*/Tn*1545*-F Tn*916*/Tn*1545*-R	GCGTGATTGTATCTCACT GACGCTCCTGTTGCTTCT	1028

[a] All these primers have been previously included in the following references (Agersø et al., 2006; De Leener et al., 2005; De Leener et al., 2004; Torres et al., 2003)

III.4.4. Results

III.4.4.1. Percentages of antimicrobial resistance and phenotypes of resistance

A total of 54 enterococcal isolates were recovered from 57 faecal samples of yellow-legged gulls included in this study and forty-two enterococci showed resistance to one or more antimicrobial agents. These isolates were identified to the species level as: *E. faecium* (52.4%), *E. faecalis* (11.9%), *E. hirae* (7.1%), and *E. durans* (2.4%). Eleven enterococci could not be identified to the

species level and they are referred as *Enterococcus* spp. Additionally, twelve enterococci isolates were susceptible to all antimicrobial agents tested (5 *E. faecium*, 4 *E. faecalis*, 2 *E. hirae*, 1 *Enterococcus* spp.)

Table III.4.2 shows the percentage of antimicrobial resistance according to the enterococcal species. It is interesting to note that all *E. faecium*, *E. hirae* and *E. durans* isolates showed resistance to tetracycline and erythromycin. Resistance to both antibiotics were also detected in almost all *E. faecalis* and *Enterococcus* spp. isolates. Resistance to ampicillin was found in 18.2% of *E. faecium* isolates and kanamycin and streptomycin resistances were present in *E. faecium* isolates (31.8% and 18.2%, respectively), and also in *Enterococcus* spp. isolates (18.2% and 27.3%, respectively). Quinupristin-dalfopristin resistance was detected in *E. faecium*, *E. hirae* and *Enterococcus* spp. isolates (27.3%, 33.3% and 45.5%, respectively) and, as expected, all *E. faecalis* were resistant to this antibiotic combination. Resistance to chloramphenicol and ciprofloxacin were detected in *E. faecalis*, *E. faecium* and *Enterococcus* spp. isolates (40%-9.1% for chloramphenicol and 36.4%-4.5% for ciprofloxacin). No gentamicin- or teicoplanin- or vancomycin-resistant enterococci were identified in this study.

Table III.4.2 | Antibiotic resistance in 42 enterococci of different species showing resistance to one or more antibiotic agents isolated from faecal samples of yellow-legged gulls

Antibiotic	Number (%) of enterococci of different species showing resistance to different antibiotics					
	E. faecium (N=22)	*E. faecalis*[a] (N=5)	*E. hirae*[a] (N=3)	*E. durans*[a] (N=1)	*Enterococcus* spp. (N=11)	Total (N=42)
Ampicillin	4 (18.2)	0	0	0	0	4 (9.5)
Gentamicin	0	0	0	0	0	0
Streptomycin	4 (18.2)	0	0	0	3 (27.3)	7 (16.7)
Kanamycin	7 (31.8)	0	0	0	2 (18.2)	9 (21.4)
Tetracycline	22 (100)	4	3	1	10 (90.9)	40 (95.2)
Erythromycin	22 (100)	4	3	1	10 (90.9)	40 (95.2)
Chloramphenicol	3 (13.6)	2	0	0	1 (9.1)	5 (11.9)
Ciprofloxacin	1(4.5)	1	0	0	4 (36.4)	6 (14.3)
Quinupristin-Dalfopristin	6 (27.3)	5	1	0	5 (45.5)	17 (40.5)
Vancomycin	0	0	0	0	0	0
Teicoplanin	0	0	0	0	0	0

[a] The number of resistant isolates is provided but not the percentage due to the low number of isolates included in the group

Table III.4.3 shows the different antimicrobial resistance phenotypes detected in our 42 isolates exhibiting resistance to one or more antibiotic agents recovered from yellow-legged gulls. The most prevalent phenotype included resistance to tetracycline and erythromycin.

III.4.4.2. Mechanisms of antibiotic resistance

The presence of antibiotic resistance genes was studied by PCR in all resistant enterococci. Forty enterococci showed tetracycline-resistance and the following resistance genes were detected:

tet(L) (10 isolates) and *tet*(M)+*tet*(L) (20 isolates). Genes specific for Tn*916*/Tn*154* transposons were identified in 14 of the 20 *tet*(M)-positive isolates and specific sequences of Tn*5397* were also identified in seven *E. faecium*, one *E. faecalis*, one *E. hirae* and one *E. durans* isolates. The presence of *erm*(B) gene was investigated in the 40 erythromycin-resistant isolates and was found in 65% of those isolates (18 *E. faecium*, three *E. faecalis*, three *E. hirae*, one *E. durans* and one *Enterococcus* spp. isolates). Three of the seventeen quinupristin-dalfopristin-resistant enterococci harboured the *vat*(E) or *vat*(D) genes (the three isolates were *E. faecium*). All the nine kanamycin-resistant isolates (seven *E. faecium* and two *Enterococcus* spp.) harboured the *aph*(3´)-IIIa gene. The *ant*(6)-Ia gene was detected in all streptomycin-resistant *E. faecium* isolates. The two chloramphenicol-resistant *E. faecalis* isolates harboured the *cat*A gene.

Table III.4.3 | Phenotypes of resistance detected among the 42 enterococci showing resistance to one or more antibiotic agents recovered from yellow-legged gulls

Bacterial species	Antimicrobial resistance patterns[a]	Number of isolates
E. faecium	TET-ERY	10
	KAN-TET-ERY	2
	TET-ERY-QD	1
	AMP-KAN-TET-ERY	1
	STR-KAN-TET-ERY	2
	TET-ERY-QD-CHL	1
	TET-ERY-QD-CIP	1
	AMP-STR-KAN-TET-ERY	1
	AMP-TET-ERY-QD-CHL	2
	STR-KAN-TET-ERY-QD	1
E. faecalis	CIP-QD	1
	TET-ERY-QD	2
	TET-ERY-QD-CHL	2
E. hirae	TET-ERY	2
	TET-ERY-QD	1
E. durans	TET-ERY	1
Enterococcus spp.	TET-ERY	4
	CIP-QD	1
	TET-ERY-QD	1
	STR-TET-ERY	1
	TET-ERY-CIP	1
	TET-ERY-QD-CHL	1
	STR-KAN-TET-ERY-CIP-QD	2

[a] AMP, ampicillin; STR, high-level streptomycin; KAN, high-level kanamycin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; QD, quinupristin-dalfopristin; CHL, chloramphenicol

III.4.4.3. Data analysis

A dendrogram with resistance profiles was elaborated in order to look for correlation among the antimicrobials tested (Figure III.4.1). It revealed that the most closely related compounds were gentamicin, teicoplanin and vancomycin (cluster 1) with 100% similarity. Ampicillin was also clustered in this group at 90% similarity. Tetracycline and erythromycin were also clustered together (cluster 2 - 100 % similarity).

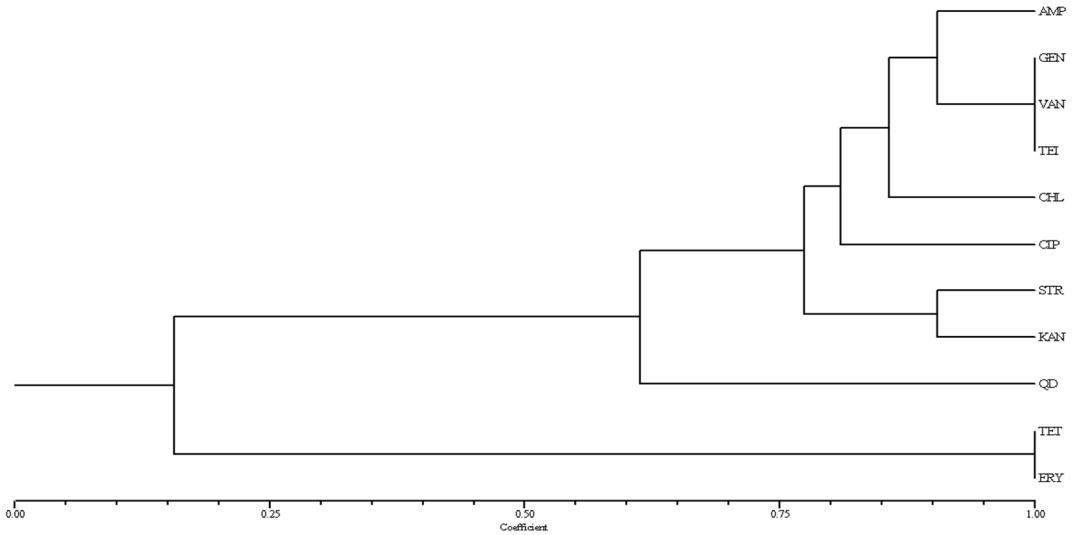

Figure III.4.1 | Dendrogram based on the numerical analysis of the resistance patterns to 11 antimicrobials of 42 enterococci using simple matching coefficient and UPGMA for clustering

III.4.5. Discussion

Selective pressure resulting from antimicrobial use is the most important force in the development of resistance (Aarestrup et al., 2000; Phillips et al., 2004; Tollefson, 2004). Enterococci, as intestinal inhabitants, are under selective pressure by every administered inhibitory antibiotic agent. For this reason, the study of antimicrobial resistance in faecal bacteria provides an important way to explore the effect of antimicrobial use.

In our report, it is shown the predominance of *E. faecium* and *E. faecalis* species among faecal enterococci. The predominance of these two species was found in other studies performed in animals (Aarestrup et al., 2000; Baldini & Selzer, 2008; Butaye et al., 2001; De Leener et al., 2005; Kojima et al., 2010; Kuhn et al., 2003; Rodrigues et al., 2002) and specifically in wild animals (Poeta et al., 2007; Poeta et al., 2005a).

In our study it is shown that *E. faecium* is the enterococcal species, which presents higher percentages of antimicrobial resistance ranging from 27.3% to 100% to quinupristin-dalfopristin, kanamycin, tetracycline and erythromycin. Lower percentages of resistance were detected to ciprofloxacin, chloramphenicol and streptomycin (4.5%-18.2%). Similar results have been found in others reports (Emaneini et al., 2008; Fracalanzza et al., 2007; Poeta et al., 2005a).

Although no gentamicin- or teicoplanin- or vancomycin-resistant isolates were identified in this study, in enterococci, multiresistance is now increasingly common and includes resistance to β-lactams, aminoglycosides (high level), and glycopeptides. The dendrogram show in Figure III.4.1 provides additional information on associations of resistance genes of enterococci. A genetic linkage between vancomycin and gentamicin resistance genes in the same plasmid is suggested. It is important to indicate the detection of four ampicillin-resistant *E. faecium* isolates. Recently, it is

relatively common to find *E. faecium* resistant to this antibiotic, due to modifications in their penicillin-binding proteins (PBP5), although this resistance phenotype has been more detected in isolates of human origin (Billstrom et al., 2008). Our results were in accordance with those found by our research group in human and animal enterococci (Poeta et al., 2007; Poeta et al., 2005a). A frequent occurrence of resistance to erythromycin and tetracycline was observed among our enterococci. As showed by dendrogram (Figure III.4.1), the high association coefficient between resistance to erythromycin and tetracycline, suggest a co-selection mechanism between them. Same results have previously been described in humans (Aarestrup et al., 2000), in poultry, swine and cattle (Kaszanyitzky et al., 2007), and also in wild animals (Poeta et al., 2005a).

Quinupristin-dalfopristin resistance was detected in 27.3% of the *E. faecium* isolates and the same results were observed by others in animal isolates (Delgado et al., 2007; Hershberger et al., 2004). A relatively low percentage of chloramphenicol resistance was found in our enterococci (11.9%) and an analogous circumstance was observed in humans, poultry and pets (Poeta et al., 2005a). In the past, chloramphenicol has been used in food-producing animals, although it was forbidden more than twenty years ago, but it is still authorized for therapeutic use in pets in Portugal.

Seventeen percent of our enterococci were resistant to streptomycin. Streptomycin resistance has also been recorded among enterococci recovered from humans in the community (Coque et al., 1995), as well as among enterococci isolates from hospitals and animals (Aarestrup et al., 2000). This report proves the occurrence of high-level kanamycin resistance among enterococci from wild animals. Kanamycin resistance was more associated with *E. faecium* in this study and the same situation is described by other authors (Aarestrup et al., 2000; Poeta et al., 2005a).

In our study, the *erm*(B), *tet*(M) and/or *tet*(L) genes have been described frequently associated together in the same strain. Tetracycline resistance genes are frequently found on the same mobile unit as macrolide resistance genes. Two mechanisms of resistance against tetracycline have been described in enterococci: an efflux-mediated mechanism encoded by *tet*(K) or *tet*(L) genes and ribosomal protection mediated by *tet*(M), *tet*(O) or *tet*(S) genes. The *erm*(B) gene is frequently linked with the *tet*(M) gene on the highly mobile conjugative transposon Tn*1545*, which predominates in clinically important Gram-positive bacteria (De Leener et al., 2004).

In this study, the *vat*(E) and the *vat*(D) genes were detected in three quinupristin-dalfopristin resistant *E. faecium* isolates. Both streptogramin-resistant genes were also detected in other studies in *E. faecium* isolates from animals and humans (Poeta et al., 2007; Robredo et al., 2000; Soltani et al., 2000).

In our study, all *E. faecium* isolates resistant to kanamycin harboured the *aph*(3´)-IIIa gene. This gene was also found in other studies (Emaneini et al., 2008; Poeta et al., 2005a; Tejedor-Junco et al., 2005).

The *ant*(6)-Ia gene, responsible for streptomycin resistance, was found in all streptomycin-resistant *E. faecium* isolates of this study. This gene was previously studied in wild boars (Poeta et al., 2007) and in humans (Klibi et al., 2006; Poeta et al., 2005a; Sepulveda et al., 2007).

The *cat*A gene, encoding a chloramphenicol acetyl-transferase, was detected in all our

chloramphenicol-resistant *E. faecalis* isolates recovered from seagulls. The *cat*A gene has been previously found in chloramphenicol-resistant enterococci of different origins by other authors (Aarestrup et al., 2000; Poeta et al., 2005a).

Previous studies have revealed the occurrence of horizontal gene transfer between bacteria that colonize animals and those that colonize humans (Murray, 1994). Antibiotic resistant bacteria such as enterococci from animals can colonize or infect the human population via contact (occupational exposure) or via the food chain. Moreover, resistance genes can be transferred from bacteria of animals to human pathogens in the intestinal microbiota of humans and so superimpose an additional load to the reservoir of resistance genes already present in humans. The proliferation of antibiotic resistance genes on transposons and plasmids in animal populations is a direct result of the widespread use of antibiotics in animals and is increased by the ease of dissemination of resistant strains between animals via faecal contact, especially among animals housed together in large groups, as with intensive farming units (Murray, 1994). Moreover, the level of resistance in the endogenous microbiota is considered a good indicator for the selection pressure exerted by antibiotic use in that population and for resistance problems to be expected in pathogens (van den Bogaard & Stobberingh, 1999). Birds are sentinel species whose plight serves as barometer of ecosystem health and alert system for detecting global environmental ills. Harmful effects seen in wildlife can be useful 'sentinel events' warning us of potential hazards for humans. This calls for integrated ecological and health hazard appraisals (Gee & Vaz, 2001).

III.4.6. Conclusion

The relatively high level of antibiotic resistance in faecal enterococci of seagulls from the Berlengas natural reserve in Portugal point out the question on the origin of the antibiotic-resistant isolates. The Berlengas natural reserve is a reservation area for the yellow-legged gulls population, this would indicate a constant exposure to antibiotics from an as-yet-uncharacterized source, or reacquisition of antibiotic-resistant bacterial strains from the surrounding environment. A potential explanation for the high occurrence of antibiotic resistance found in the seagulls enterococci population could be the transfer of antibiotic-resistant strains. It could be also due to possible contact with humans because people can move to these natural areas or vice versa, and very sporadic contact could exist. In the early eighties, with the proliferation of open rubbish dumps (urban solid waste that includes, predominantly, domestic waste) along the Peniche coast the population of seagulls increased. Refuse dumps that could be a source of antibiotic-resistant bacteria provide abundant food supply, particularly during the inter-breeding period, while discarded fish represent the greatest part of the diet. Fish microflora is strongly affected by natural occurring factors, such as environment, human made factors such as farming, industries and sewage treatment plants that are in contact with

marine habitats. According with our results, enteric enterococci of gulls show frequently antibiotic resistance to several antibiotics and contain antibiotic resistance genes. It can not be discarded that pollution of water with antibiotics, resistant bacteria and/or resistance genes (e.g. faecal material of animals or humans) could had been a contributing factor for this detection. These facts might be involved in the acquisition and dissemination of antibiotic resistance genes even in the absence of direct antibiotic pressure. The seagulls population on Berlengas natural reserve provides an opportunity to monitor the impact of antibiotic use on the natural environment and can serve as a sentinel for antibiotic pressure from the surrounding farm and urban environments. The significant presence of antibiotic resistance genes in yellow-legged gulls will allow tracking of the flow of resistance genes and the identification of the origins of these determinants in the environment. In this study, the seagulls population can serve as a sentinel and was chosen as the model system to evaluate antibiotic resistance in a wild environment. The study suggests that seagulls are an important host reservoir of resistant enterococci strains, probably reflecting the presence of such strains in their sources of food and/or water. Although gulls do not naturally come into contact with antibiotics, these birds can be infected with resistant enterococci and potentially serve as their reservoirs, vectors and bioindicators in the environment.

III.4.7. Acknowledgements

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III.4.8. References

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CHAPTER IV

GENOMIC CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT *E. COLI* AND ENTEROCOCCI ISOLATES OF WILD ANIMALS WITH ANTIMICROBIAL SELECTIVE PLATES

CHAPTER IV

IV.1. Poeta P, Radhouani H, Igrejas G, et al. (2008) **Seagulls of the Berlengas natural reserve of Portugal as carriers of faecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases.**

IV.2. Radhouani H, Pinto L, Coelho C, et al. (2010) **Detection of *Escherichia coli* harbouring extended-spectrum beta-lactamases of the CTX-M classes in faecal samples of common buzzards (*Buteo buteo*).**

IV.3. Radhouani H, Igrejas G, Gonçalves A, et al. (2013) **Molecular characterization of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates from red foxes in Portugal.**

IV.4. Radhouani H, Pinto L, Coelho C, et al. (2010) **MLST and a genetic study of antibiotic resistance and virulence factors in *van*A-containing *Enterococcus* from buzzards (*Buteo buteo*).**

IV.5. Radhouani H, Igrejas G, Carvalho C, et al. (2011) **Clonal Lineages, antibiotic resistance and virulence factors in vancomycin-resistant enterococci isolated from fecal samples of red foxes (*Vulpes vulpes*).**

IV.1. Seagulls of the Berlengas natural reserve of Portugal as carriers of faecal *Escherichia coli* harboring CTX-M and TEM extended-spectrum beta-lactamases

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IV.1.1. Abstract

Escherichia coli isolates containing the following extended-spectrum beta-lactamases have been detected in 11 of 57 faecal samples (19.3%) in Berlengas Island seagulls: TEM-52 (eight isolates), CTX-M-1 (one isolate), CTX-M-14a (one isolate), and CTX-M-32 (one isolate). Most of the extended-spectrum beta-lactamase-positive isolates harbored class 1 or class 2 integrons, which included different antibiotic resistance gene cassettes.

IV.1.2. Introduction

The emergence and wide dissemination of extended-spectrum beta-lactamases (ESBLs) among clinical *Escherichia coli* isolates in hospitals in recent years are of great concern and represent a problem for the treatment of infectious diseases (Paterson & Bonomo, 2005). It has also been reported that *E. coli* isolates containing ESBLs, mostly of the CTX-M class, are frequently detected in community patients (Cantón et al., 2008) and have also been found in food producing animals and household pets (Blanc et al., 2006; Briñas et al., 2002; Carattoli, 2008; Costa et al., 2004; Girlich et al., 2007; Kojima et al., 2005; Meunier et al., 2006; Teale et al., 2005). Moreover, a previous report identified ESBLs in faecal *E. coli* isolates of wild animals (Costa et al., 2006), mainly in birds of prey, but seagulls were not included in that study. ESBLs seem to be widely distributed in bacteria of different ecosystems, although more information is needed, especially for wild ecosystems.

The purpose of our study was to analyze the carriage of ESBL-containing *E. coli* isolates in faecal samples of Berlengas Island seagulls and also to characterize the type of ESBLs and the phylogenetic groups of isolates. Berlengas Island is part of the Berlengas Natural Reserve, located 5.7 miles from the Portuguese coast, and it belongs to the National Network of Protected Areas. Fishermen inhabited the island in the past, but currently nobody lives there year round, although some tourists visit the island and a few people stay for vacations. Diverse species of seagulls make their nests on this island; in the last few years the population of seagulls has increased significantly and is considered a true plague (Morais et al., 1995).

IV.1.3. Material and methods

Fifty-seven fresh seagull faecal droppings were obtained in different areas of the Berlengas Island during September 2007 and were tested for the presence of ESBL-containing *E. coli* isolates. Faecal samples were seeded in Levine agar plates supplemented with cefotaxime (CTX; 2 μg/mL), and colonies with typical *E. coli* morphology were selected and identified by classical biochemical methods and by the API 20E system (bioMérieux, La Balme Les Grottes, France). Susceptibility of the recovered *E. coli* isolates to 16 antibiotics (ampicillin, amoxicillin plus clavulanic acid, cefoxitin, CTX, ceftazidime, aztreonam, imipenem, gentamicin, amikacin, tobramycin, streptomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole-trimethoprim, tetracycline, and chloramphenicol) was tested by the disk diffusion method (CLSI, 2007). *E. coli* ATCC 25922 was used as a quality control strain. Broad-spectrum cephalosporin-resistant isolates were selected for further studies (one isolate per sample), and they were screened for ESBL production according to the CLSI criteria (CLSI, 2007).

The presence of genes encoding TEM, SHV, OXA, CTX-M, and CMY type beta-lactamases was studied by specific PCRs (Bertrand et al., 2006; Jouini et al., 2007; Stapleton et al., 1999). All obtained amplicons were sequenced on both strands, and sequences were compared with those included in the GenBank database and in the website http://www.lahey.org /Studies/ to identify the beta-lactamase genes. The genetic environment of $bla_{\text{CTX-M}}$ genes was also tested by PCR and by sequencing with previously reported primers (Eckert et al., 2006; Lartigue et al., 2004; Saladin et al., 2002).

The presence of other antibiotic resistance genes, associated with chloramphenicol (*cml*A), tetracycline (*tet*(A) and *tet*(B)), streptomycin (*aad*A), and sulfonamide (*sul*1, *sul*2, and *sul*3) resistance, among our isolates was also analyzed by PCR and sequencing (Sáenz et al., 2004). The presence of the *intI*1 and *intI*2 genes, encoding class 1 and 2 integrases, respectively, and the composition of the variable regions of class 1 and 2 integrons were studied by PCR and sequencing (Sáenz et al., 2004). The identification of the major phylogenetic groups among our isolates was determined by PCR (Clermont et al., 2000). Positive and negative controls from the bacterial collection of the University of La Rioja, Logroño, Spain, were used in all assays.

IV.1.4. Results and discussion

E. coli isolates were detected in Levine CTX plates from 11 of the 57 (19.3%) faecal samples studied. All 11 isolates obtained from these samples were intermediate or resistant to CTX and/or ceftazidime and had a positive screening test for ESBL production. Only 1 of the 11 *E. coli* isolates (GV-10) showed resistance to amoxicillin plus clavulanic acid. The beta-lactamase genes detected in these isolates were the following (numbers of isolates are in parentheses): $bla_{\text{TEM-52}}$ (8), $bla_{\text{CTX-M-1}}$ plus $bla_{\text{OXA-1}}$ (1), $bla_{\text{CTX-M-14a}}$ (1), and $bla_{\text{CTX-M-32}}$ (1) (Table IV.1.1). It is interesting that 73% of the ESBL-positive isolates of seagulls harbored the $bla_{\text{TEM-52}}$ gene and that 27% of the isolates harbored the $bla_{\text{CTX-M}}$ gene. A high prevalence of TEM-52 has also been recently observed in *E. coli* isolates from healthy food-producing animals and chicken meat products from Portugal (Machado et al., 2008).

Different genetic environments surrounding the $bla_{\text{CTX-M}}$ genes were detected (Figure IV.1.1). The sequence of the fragment obtained by PCR upstream of the $bla_{\text{CTX-M-1}}$ gene in the *E. coli* GV-23 strain revealed the presence of a region of the IS*26* transposase flanking a partially truncated IS*Ecp1* followed by an intergenic region; this whole structure has been previously found in *E. coli* (Jouini et al., 2007; Saladin et al., 2002). The presence of IS*Ecp1* and IS*903* surrounding the $bla_{\text{CTX-M-14a}}$ gene in *E. coli* GV-10 was identified, and the genetic environment of the $bla_{\text{CTX-M-32}}$ gene detected in *E. coli* GV-12 included IS*Ecp1*/IS*5* upstream of the *bla* gene and *orf477* downstream, as also detected by others (Vinué et al., 2008).

A variety of resistance genes (*cml*A, *tet*(A), *tet*(B), *aad*A, *sul*1, *sul*2, and *sul*3) were observed among our ESBL-producing *E. coli* isolates (Table IV.1.1). Five isolates harbored class 1 integrons with the following gene cassettes in their variable regions: *dfr*A1 plus *aad*A1 (two isolates), *sat* plus *psp* plus *aad*A2 (one isolate), *sat* plus *aad*A1 (one isolate), and $bla_{\text{OXA-1}}$ plus *aad*A1 (one isolate). Five isolates harbored class 2 integrons, and the gene cassette arrangement *dfr*A1 plus *sat* plus *aad*A1 was identified in all of them. *E. coli* GV-8 contained simultaneously class 1 and 2 integrons. Eight of the ESBL-positive isolates corresponded to the A and B1 phylogenetic groups, two isolates corresponded to the D group, and only one $bla_{\text{TEM-52}}$ isolate was assigned to the B2 phylogenetic group (Table IV.1.1). Previous studies have reported the association of *E. coli* isolates of the B2 group with extraintestinal infections (Picard et al., 1999), and the faecal origin of our isolates could explain the low prevalence of this phylogroup.

IV.1.5. Conclusion

It is important to note the high prevalence and moderate diversity of ESBLs detected in faecal *E. coli* from seagulls that inhabit a natural reserve, as is the case for Berlengas Island. As previously indicated, the population of seagulls on an island that is not too far away from the Portuguese coast has significantly increased in recent years. The possibility that these animals eat the remains of human food cannot be excluded. This study gives new evidence for the wide dissemination of ESBLs in *E. coli* isolates from wild animals, as is the case for seagulls. More studies of this nature should be performed in the future to analyze the prevalence of this type of resistant bacteria in different ecosystems.

IV.1.6. Acknowledgements

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Table IV.1.1 | Characteristics of the ESBL-positive faecal *E. coli* isolates recovered from seagulls of Berlengas island in Portugal

E. coli Isolate	Phylogenetic group	Phenotype of antimicrobial resistance for non-beta-lactams[a]	Type of ESBLs	Integrons				Other genes detected
				*intI*1	Gene cassettes in class 1 integrons	*intI*2	Gene cassettes in class 2 integrons	
GV-5	D	NAL-CIP-TET-STR-SXT-CHL	TEM-52	+	*dfr*A1+*aad*A1	-		*tet*(A), *sul*1, *sul*3, *cml*A
GV-6	B1	NAL-CIP-TET-STR	TEM-52	-		+	*dfr*A1+*sat*+*aad*A1	*tet*(A)
GV-8	B2	NAL-CIP-TET-STR-SXT-CHL	TEM-52	+	*sat*+*psp*+*aad*A2 [b]	+	*dfr*A1+*sat*+*aad*A1	*tet*(A), *sul*2, *sul*3, *cml*A
GV-9	B1	NAL-CIP-TET-STR- SXT-CHL	TEM-52	+	*dfr*A1+*aad*A1	-		*tet*(A), *sul*1, *sul*2, *sul*3, *cml*A
GV-33	A	NAL-TET	TEM-52	-		-		*tet*(B)
GV-51	B1	NAL-CIP-TET-SXT	TEM-52	-		+	*dfr*A1+*sat*+*aad*A1	*tet*(A), *sul*2
GV-52	A	NAL CIP-TET	TEM-52	-		+	*dfr*A1+*sat*+*aad*A1	*tet*(A)
GV-54	D	NAL-CIP-TET-STR-SXT	TEM-52	-		+	*dfr*A1+*sat*+*aad*A1	*tet*(B), *sul*2
GV-23	A	TET-CHL	CTX-M-1	+	bla_{OXA-1}+*aad*A1	-		*tet*(B), *sul*1
GV-10	A	NAL-TET-STR	CTX-M-14a	-		-		*tet*(B), *aad*A
GV-12	B1	NAL-CIP-TET-STR	CTX-M-32	+	*sat*+*aad*A1[b]	-		*tet*(B)

[a] TET, tetracycline; CHL, chloramphenicol; NAL, nalidixic acid; CIP, ciprofloxacin; STR, streptomycin; SXT, trimethoprim/sulfamethoxazole
[b] The *qacE*Δ +*sul1* 3' CR was absent in two *intI*1-positive *E. coli* isolates containing bla_{TEM-52} and $bla_{CTX-M-32}$

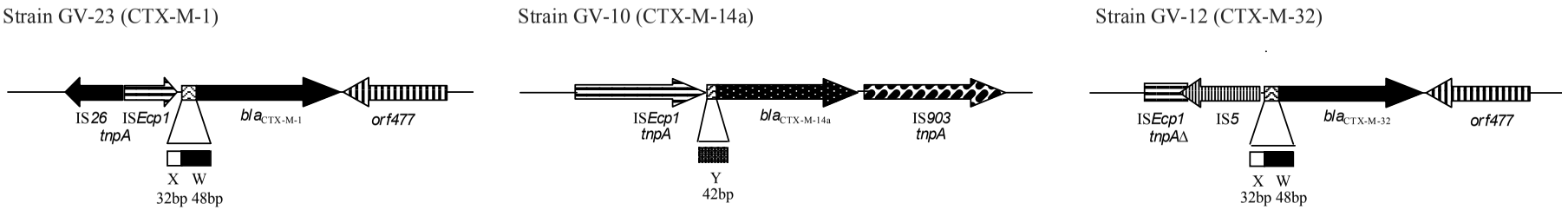

Figure IV.1.1 | Genetic environment of bla_{CTX-M} genes in three *E. coli* isolates of seagulls

IV.1.7. References

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IV.2. Detection of *Escherichia coli* harbouring extended-spectrum β-lactamases of the CTX-M classes in faecal samples of common buzzards (*Buteo buteo*)

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IV.2.1. Abstract

Extended-spectrum beta-lactamase (ESBL) containing *E. coli* isolates have been detected in five of thirty three faecal samples (15%) of common buzzards (*Buteo buteo*) of Portugal, when samples were inoculated in Levine-agar plates supplemented with cefotaxime. Two ESBL-positive *E. coli* isolates per sample showing different phenotypic profiles were selected for further studies. The ESBLs detected by PCR and sequencing in these 10 ESBL-positive isolates were as follows: CTX-M-1+TEM-1 (3 isolates), and CTX-M-32+TEM-1 (7 isolates). The $bla_{\text{CTX-M-1}}$ gene was surrounded by IS*Ecp1* and *orf477*, and this last sequence was also found downstream of $bla_{\text{CTX-M-32}}$ gene. All these isolates contained the *intI*1 gene, encoding the integrase of class 1 integrons but were negative for *intI*2 gene, encoding the integrase of class 2 integrons. The *tet*(A) or *tet*(B) genes were present in all isolates of this study. The *aad*A gene was found in the six streptomycin-resistant isolates. The *aac*(3)-IV gene was detected in the two gentamicin-resistant isolates. The *sul*1 and/or *sul*2 and/or *sul*3 genes were found in all of the SXT-resistant isolates. Three isolates carried simultaneously *sul*1 and *sul*2 genes, one isolate contained both s*ul*2 and *sul*3 genes, and six isolates *sul*1, *sul*2 and *sul*3 genes. The phylogenetic groups detected in ESBL-positive *E. coli* isolates were: B1 (4 isolates), B2 (4), and A (2). All $bla_{\text{CTX-M-1}}$ isolates were classified in B1 phylogroup, and 4 of 7 $bla_{\text{CTX-M-32}}$ isolates in B2 phylogroup. The intestinal microbiota of common buzzards is a reservoir of ESBLs of the CTX-M group.

IV.2.2. Introduction

In recent years, infections due to *Escherichia coli* harbouring extended-spectrum β-lactamases (ESBLs) of the CTX-M classes have dramatically increased among human populations, particularly in the community setting (Livermore et al., 2007). There exist just a few previous reports in which ESBLs were detected in faecal *E. coli* isolates of wild animals, (Poeta et al., 2008) but, to our knowledge, never in faecal *E. coli* isolates of buzzards (*Buteo buteo*).

IV.2.3. Material and methods

Thirty-three faecal samples from buzzards of Portugal were recovered from September 2007 to February 2008 and were studied for the presence of ESBL-producing *E. coli* isolates. All the faecal samples were collected individually from each buzzard and obtained in collaboration with CRATAS (Centre of Collecting, Welcome and Handling of Wild Animals). This centre is located in the University of Trás-os-Montes and Alto Douro and receives injured animals. None of the buzzards had been previously fed by humans or had received antibiotics. Most of the animals inhabited the Pêneda Gêres Natural Park or other rural conservation areas of Portugal. Faecal samples were screened for the presence of ESBLs using Levine agar (Oxoid Limited, UK) supplemented with 2 μg/mL cefotaxime (Levine–CTX) (Sigma–Aldrich, USA). Two colonies with typical *E. coli* morphology were selected and identified by classical biochemical methods (Gram, catalase, oxidase, indole, Methyl Red–Voges–Proskauer, citrate and urease) and by the API 20E system (BioMérieux, La Balme Les Grottes, France) from each positive faecal sample. Susceptibility to 16 antibiotics (ampicillin, amoxicillin/clavulanic acid, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, gentamicin, amikacin, tobramycin, streptomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole/trimethoprim, tetracycline and chloramphenicol) (Oxoid Limited, UK) was determined by the CLSI disc diffusion method (CLSI, 2007) for all recovered *E. coli* isolates. *E. coli* ATCC 25922 was used as a quality-control strain. Isolates resistant to third-generation cephalosporins (i.e. cefotaxime or ceftazidime) were selected for further studies (one per faecal sample, or two if they presented different phenotypes of antibiotic resistance). The double disc diffusion test (cefotaxime, ceftazidime and aztreonam in the presence or absence of amoxicillin/clavulanic acid) was performed to detect ESBL production.

The presence of genes encoding TEM-, SHV-, OXA- and CTX-M-type β-lactamases was studied by specific PCR (Poeta et al., 2008), and positive amplicons were sequenced to determine the specific type of β-lactamase gene. The genetic environment of $bla_{\text{CTX-M}}$ genes was also studied by PCR and sequencing in all $bla_{\text{CTX-M}}$ containing isolates using primers previously reported (Poeta et al., 2008). In addition, *IS*903, IS*Ecp1* and *orf477* were also amplified by PCR (Eckert et al., 2006). The following resistance genes were also studied by PCR (Poeta et al., 2008): *tet*(A) and *tet*(B) (in

tetracycline-resistant isolates), *aad*A (in streptomycin-resistant isolates), *aac*(3)-II and *aac*(3)-IV (in gentamicin-resistant isolates), and *sul*1, *sul*2 and *sul*3 (in SXT-resistant isolates). The presence of the *intI*1 and *intI*2 genes, encoding class 1 and 2 integrases, respectively and the detection of phylogenetic groups of *E. coli* were studied by PCR (Poeta et al., 2008).

IV.2.4. Results and discussion

E. coli colonies were isolated from 5 of the 33 (15.2 %) faecal samples in the Levine-CTX screen. Two *E. coli* isolates from each positive sample were recovered, showing different phenotypic and genomic profiles. All 10 of these isolates exhibited a resistant phenotype to cefotaxime and/or ceftazidime, and gave a positive ESBL production test. The β-lactamase genes detected in the ESBL-positive *E. coli* isolates were the following: $bla_{\text{CTX-M-32}}$ + $bla_{\text{TEM-1}}$ (seven isolates); and $bla_{\text{CTX-M-1}}$ + $bla_{\text{TEM-1}}$ (three isolates) (Table IV.2.1). The high prevalence of $bla_{\text{CTX-M-32}}$ in commensal *E. coli* isolates of buzzards in this study is remarkable (15.2% of total buzzards), as this gene is not frequently found in animal isolates, having been reported in only a few studies previously (Poeta et al., 2008; Politi et al., 2005). Additionally, some of the birds were colonized with both CTX-M-1- and CTX-M-32-producing *E. coli*. The *orf477* sequence was detected downstream of the $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$ genes, and IS*Ecp1* was found upstream of the $bla_{\text{CTX-M-1}}$ gene in all our isolates; similar molecular arrangements incorporating CTX-M genes have been previously reported (Eckert et al., 2006; Poeta et al., 2008). The association of insertion sequences (e.g. IS*Ecp1*) upstream of the CTX-M β-lactamase genes may be involved in their dissemination and expression. Our CTX-M-containing isolates were all multiresistant, with resistance to tetracycline and sulfamethoxazole/ trimethoprim due to *tet*(A) [associated or not with *tet*(B)] and different combinations of the three *sul* genes, respectively. In addition, the *aad*A gene was detected in the six streptomycin-resistant isolates and the *aac*(3)-IV gene in the two gentamicin-resistant isolates (Table IV.2.1). All the $bla_{\text{CTX-M-1}}$-producing isolates were classified in the B1 phylogroup. The $bla_{\text{CTX-M-32}}$-producing isolates belonged to the B2 (four isolates), A (two isolates) or B1 (one isolate) phylogroups. The B2 phylogroup has been associated in previous reports with more-virulent isolates and has also been linked with some specific mechanisms of resistance, as is the case of the β-lactamase CTX-M-15 (Clermont et al., 2000). The possible association of $bla_{\text{CTX-M-32}}$ in isolates of the B2 phylogroup should be tracked in the future.

IV.2.5. Conclusion

This is the first time, to our knowledge, that CTX-M-producing *E. coli* isolates have been detected in buzzards. Buzzards are carnivorous birds, and they can fly large distances searching for food and territory. It is possible that these feeding habits could expose them to faecal material of

farm animals or even of humans. This might explain the acquisition and dissemination of bacteria harbouring antibiotic resistance genes in the buzzard population, even in the absence of direct antibiotic pressure. This study highlights that ESBLs are found in ecosystems other than those closely related to humans or containing obvious antibiotic resistance selection pressures. More studies should be carried out in the future with different kinds of wild animals to confirm the dissemination of ESBLs in other ecosystems and animal populations.

Table IV.2.1 | Characteristics of the ESBL-positive faecal *E. coli* isolates recovered from buzzards

E. coli isolate	Phylogenetic group	Phenotype of antimicrobial resistance for non-β-lactams	β-lactamases detected	Environment of bla_{CTX-M} genes	Integrons *intI*1	Other resistance genes detected
BU-10A	B2	NAL, TET, SXT	TEM-1+ CTX-M-32	*orf477*	+	*tet*(A), *sul*1, *sul*2, *sul*3
BU-10B	B1	NAL, TET, STR, SXT	TEM-1+ CTX-M-1	*orf477*, IS*Ecp1*	+	*tet*(A), *aad*A, *sul*1, *sul*2, *sul*3
BU-21A	B1	AK, TET, STR, SXT	TEM-1+ CTX-M-1	*orf477*, IS*Ecp*1	+	*tet*(A), *aad*A, *sul*1, *sul*2, *sul*3
BU-21B	B1	TET, STR, SXT	TEM-1+ CTX-M-32	*orf477*	+	*tet*(A), *aad*A, *sul*1, *sul*2, *sul*3
BU-22A	B1	NAL, TET, SXT	TEM-1+ CTX-M-1	*orf477*, IS*Ecp1*	+	*tet*(A), *tet*(B), *sul*1, *sul*2
BU-22B	B2	GEN, TET, SXT	TEM-1+ CTX-M-32	*orf477*	+	*aac*(3)-IV, *tet*(A), *tet*(B), *sul*1, *sul*2, *sul*3
BU-36A	A	GEN, NAL, TET, STR, SXT	TEM-1+ CTX-M-32	*orf477*	+	*aac*(3)-IV, *tet*(A), *aad*A, *sul*1, *sul*2, *sul*3
BU-36B	B2	TET, STR, SXT	TEM-1+ CTX-M-32	*orf477*	+	*tet*(A), *tet*(B), *aad*A, *sul*2, *sul*3
BU-41A	B2	TET, SXT	TEM-1+ CTX-M-32	*orf477*	+	*tet*(A), *aad*A, *sul*1, *sul*2
BU-41B	A	TOB, NAL, CIP, TET, STR, SXT	TEM-1+ CTX-M-32	*orf477*	+	*tet*(A), *sul*1, *sul*2

IV.2.6. Acknowledgements

We thank the CRATAS (Centre of Collecting, Welcome and Handling of Wild Animals) for their contribution to the collection of samples.

IV.2.7. References

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IV.3. Molecular characterization of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates from red foxes in Portugal

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IV.3.1. Abstract

The presence of broad-spectrum-cephalosporin-resistant *Escherichia coli* isolates, the implicated mechanisms of resistance and virulence factor genes were investigated in red fox (*Vulpes vulpes*) in Portugal. Cefotaxime-resistant *E. coli* isolates have been isolated from 2 of 52 faecal samples (4%), being both ESBL-producers. The β-lactamase genes found in the two isolates were $bla_{SHV-12} + bla_{TEM-1b}$. The *tet*(A) and *sul*2 genes were also detected in these isolates, together with the non-classical class 1 integron (*intI*1-*dfr*A12-*orf*F-*aad*A2-*cml*A1-*aad*A1-*qacH*-IS*440*-*sul*3) with the PcH1 promoter. The two isolates belonged to the phylogroup A. Amino acid changes in GyrA (S83L+D87G) and ParC (S80I) proteins were identified in our study. Concerning MLST typing, both isolates were assigned to ST1086, never found before in wild animals and they presented closely-related PFGE patterns. This study reveals the presence of ESBL-producing *E. coli* isolates, in a wild ecosystem, which could be disseminated through the environment.

IV.3.2. Introduction

The production of extended-spectrum β-lactamases (ESBLs) by Enterobacteriaceae, in particular by *Escherichia coli*, has been increasingly reported in the last few years in healthy animals and has caused a major concern in diverse countries including Portugal, being frequently implicated in human infections (Paterson & Bonomo, 2005). Some previous studies do exist in which ESBL-producing *E. coli* isolates were detected in wild animal faecal samples, allowing the scientific community for a better knowledge of the problem of antimicrobial resistance in the wildlife and the consequences of this aspect in different ecosystems. The purpose of our work was to analyze the faecal carriage of ESBL-producing *E. coli* isolates in red foxes (*Vulpes vulpes*) in Portugal, to identify the type of ESBLs, to detect the presence of other antimicrobial resistance markers, as well as to detect the phylogenetic groups and virulence genes in these bacteria.

IV.3.3. Material and methods

Fifty-two faecal samples of red foxes were studied in this report. Samples were collected (one sample per individual) from February-2008 to March-2009 in North of Portugal during hunts of red foxes. This kind of hunting is organized all of the years during a short period of time having like main objective the ecological control of the animal population. The animals were obtained in collaboration with the Portuguese National Corporation of Forest Rangers. As far as we know, none of the animals had been previously fed by humans or had received antimicrobials. Samples were seeded in Levine agar plates supplemented with cefotaxime (2 mg/L), and colonies with typical *E. coli* morphology (one per sample) were selected and identified by the API 20E system (BioMérieux, La Balme Les Grottes, France).

Susceptibility of the *E. coli* isolates to 16 antimicrobial agents [ampicillin, amoxicillin plus clavulanic acid, cefoxitin, cefotaxime (CTX), ceftazidime, aztreonam, imipenem, gentamicin, amikacin, tobramycin, streptomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole-trimethoprim (SXT), tetracycline, and chloramphenicol] was performed by the disk diffusion method (CLSI, 2010). *E. coli* ATCC 25922 was used as a quality-control strain. ESBL-phenotypic detection was carried out by double-disk diffusion test (CLSI, 2010).

The presence of genes encoding TEM, OXA, SHV, and CTX-M type beta-lactamases were studied by PCR and positive amplicons were sequenced to determine the specific type of β-lactamase gene (Gonçalves et al., 2012). The following antimicrobial resistance genes were studied by PCR: *tet*(A), *tet*(B), *aad*A, *str*A-*str*B, *aac*(3)-II, *aac*(3)-IV, and *cml*A (Gonçalves et al., 2012). The presence of the *intI*1 and *intI*2 genes, encoding class 1 and 2 integrases, respectively and their variable region were also analysed by PCR and sequencing (Gonçalves et al., 2012). The presence of *sul*1, *sul*2 and *sul*3 genes was studied by PCR and *qacH*-IS*440* region, associated with *sul*3 and non-classical class 1 integron, was analysed by a primer-walking PCR strategy (Vinué et al., 2010).

The quinolone resistance-determining region of *gyr*A and *par*C genes were amplified by PCR, sequence and examined in all quinolone-resistant isolates (Gonçalves et al., 2012).

The presence of genes encoding different virulence factors (*fim*A, *pap*GIII, *stx*, *cnf*1, *pap*C and *aer*) was also verified by PCR (Ruiz et al., 2002).

The isolates of *E. coli* were assigned to one of the four main phylogenetic groups A, B1, B2 and D, following the PCR strategy previously published based in the presence or absence of *chu*A, *yja*A genes or DNA fragment TSPE4.C2 (Clermont et al., 2000).

Isolates were analyzed by multilocus sequence typing (MLST) by using the *E. coli* MLST scheme (http://mlst.ucc.ie/mlst/dbs/Ecoli), which is based on sequencing of internal regions of the 7 housekeeping genes *adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A, and *rec*A (Wirth et al., 2006). Positive and negative controls from the collection of strains of the University of Trás-os-Montes and Alto Douro (Portugal) and of the University of La Rioja (Spain) were included in all PCR assays.

The clonal relationship between the ESBL-producing isolates was determined by pulsed-field gel electrophoresis (PFGE) (Gautom, 1997). Patterns obtained were analyzed according to previously reported criteria (Tenover et al., 1995).

IV.3.4. Results

Results showed that CTX-resistant *E. coli* isolates were present in two of the 52 samples of our study, representing 4% of the total faecal samples. Concerning these two animals, they were hunted on different days from February-2008 to March-2009 but they inhabit in the same geographical area.

CTX-resistant isolates of both samples (C5478 and C5482) showed a positive screening test for ESBL-production. The two isolates showed resistance to ampicillin, amoxicillin+clavulanic acid, CTX, ceftazidime, aztreonam, tetracycline, sulfamethoxazole/trimethoprim, streptomycin, nalidixic acid, ciprofloxacin and chloramphenicol. PFGE analysis showed that the both ESBL-positive isolates share closely related profiles.

The β-lactamase genes detected in the two ESBL-producing *E. coli* isolates of our study were $bla_{\text{SHV-12}} + bla_{\text{TEM-1b}}$. The two isolates harboured the genes *tet*(A) and *sul*2 and contained the non-classical class 1 integron (*intI*1-*dfr*A12-*orf*F-*aad*A2-*cml*A1-*aad*A1-*qacH*-IS*440*-*sul*3) with the PcH1 promoter. The presence of two amino acid changes in GyrA (S83L+D87G) and one in ParC (S80I) were responsible for the ciprofloxacin and nalidixic acid resistance phenotype detected in our two isolates. Concerning MLST typing, both *E. coli* isolates were assigned to the sequence type ST1086. Furthermore, these isolates were typed as phylogroup A and carried the *fim*A virulence gene.

IV.3.5. Discussion

To our knowledge, the present study represents the first molecular report of ESBL-producing *E. coli* isolates in red fox faecal samples. Almost 4% of the faecal samples of red foxes revealed ESBL-producing *E. coli* isolates. This percentage is lower than that found by others in different wild animals (fox, owl, deers, and birds of prey) (12.5%) (Costa et al., 2006). Other study revealed the presence of important sources of colonization by ESBL-producing *E. coli* isolates in red foxes in Germany (Grobbel et al., 2012). Furthermore, Literak and co-workers (Literak et al., 2010b) showed the presence of resistant *E. coli* isolates, particularly in ruminants, but also in red fox (12%) in Slovakia.

The $bla_{\text{SHV-12}}$ gene identified in our study, are frequently detected in Spain and Portugal (Coque et al., 2008). Besides the $bla_{\text{CTX-M}}$, $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-52}}$ were the most often detected in wild animals (Guenther et al., 2011). Moreover, in the last decade, SHV-12-producing *E. coli* have been increasingly reported in food-producing animals, food and humans (EFSA Panel on Biological Hazards (BIOHAZ), 2011). Foxes do enter human habitat and can scavenge human garbage, so can easily be in contact with food that could be contaminated by resistant isolates of human origin, or poultry and domestic animals also could be the source (Literak et al., 2010b). These findings suggest the presence of significant sources of colonization by SHV-12-producing *E. coli* in wildlife ecosystem.

In the present study, the occurrence of the non-classical class 1 integron (*intI*1-*dfr*A12-*orf*F-*aad*A2-*cml*A1-*aad*A1-*qacH*-IS*440*-*sul*3) has been revealed. Integrons are elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination. The detection of integrons may be attributed to its wide spread distribution in Gram-negative microorganism colonizing both humans and animals, and they could be more widespread in nature than originally thought (Literak et al., 2010b); the detection of these structures in our isolates could be a reflect of this point.

The association of beta-lactam resistance with tetracycline, nalidixic acid, streptomycin and trimethoprim resistances observed in our study has also been previously demonstrated in isolates of wild animals (Literak et al., 2010a) and also of human origin (Sáenz et al., 2004). Moreover, the substitutions S83L, D87G and S80I observed in our report, were also found in wild animals (Costa et al., 2009). To our knowledge, the sequence type ST1086 was never found before in wild animals. The two isolates were closely related by PFGE and, therefore, the occurrence of resistance might be due to the dissemination of a specific clone.

The detection of few virulence determinants in our study may be due to the fact that our isolates belonged to the low-virulence phylogenetic group A. Furthermore, the *fim*A virulence gene is frequently detected among commensal *E. coli* isolates (Dobrindt et al., 2003).

IV.3.6. Conclusion

Due to the common occurrence of red foxes in Europe, and to their predatory nature, these wild animal animals harbour resistance genes and so they could spread resistant bacteria throughout the environment. Therefore, natural environments represent reservoirs of antibiotic resistance genes and the results obtained in this study are a reflex of this situation. The evolution of this type of resistance in wild animals and in general in natural ecosystems is of great importance in human medicine. The incidence of antimicrobial resistant genes in red foxes may be due to their diet as these wild animals usually hunt wild rabbits, small rodents and birds. Foxes are on top of the food chain, perhaps accumulating multiresistant bacteria from their prey (Grobbel et al., 2012). It is also important to highlight that some studies reported the presence of antimicrobial resistant *E. coli* isolates in wild rabbits (Silva et al., 2010), wild rodents (Guenther et al., 2010) and wild birds (Radhouani et al., 2010). All these evidences may contribute in the acquisition and spread of antimicrobial resistant bacteria even in the absence of direct antimicrobial pressure and thus explain the presence of ESBL-producing *E. coli* isolates. More studies should be performed in the future to analyze the flow and evolution of broad spectrum-cephalosporin-resistant *E. coli* isolates in different ecosystems in order to assess its implication in human health.

IV.3.7. Supplementary material

Supplementary material (Table S1) regarding this manuscript is available at Arch Microbiol online (http://link.springer.com/article/10.1007%2Fs00203-012-0853-7).

IV.3.8. Acknowledgements

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IV.4. MLST and a genetic study of antibiotic resistance and virulence factors in *van*A-containing *Enterococcus* from buzzards (*Buteo buteo*)

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IV.4.1. Abstract

Aims: To analyse the occurrence of faecal carriage of vancomycin-resistant enterococci (VRE) in *Buteo buteo* and to study the associated resistance and virulence genes.

Methods and Results: The presence of VRE was investigated in 33 faecal samples of *B. buteo*. Samples were seeded in Slanetz–Bartley agar plates supplemented with vancomycin for VRE recovery. Genes encoding antimicrobial resistance and virulence were studied by polymerase chain reaction. Vancomycin-resistant *Enterococcus faecium* isolates were characterized by multilocus sequence typing. VRE with an acquired mechanism of resistance (*van*A genotype) were detected in 9% of samples analysed (*E. faecium* and *E. durans*). In addition, 27% of samples contained VRE with an intrinsic mechanism of resistance (*E. gallinarum*, *vanC*1). All *van*A-containing isolates showed resistance to tetracycline and erythromycin and harboured the *tet*(M) and/or *tet*(L) genes, in addition to the *erm*(B) gene. The *vat*(E) and/or *vat*(D), *cat*A and *aph*(3′)-IIIa genes were identified in quinupristin–dalfopristin-, chloramphenicol-, and kanamycin-resistant *van*A-containing strains, respectively. The sequence types ST273 and ST5 were identified in two *van*A-positive *E. faecium* isolates, and the presence of *hyl*, *gel*E, *cyl*A, *cyl*L and *cyl*M virulence genes and gelatinase activity were identified in *E. faecium* ST5 strain.

Conclusions: The intestinal tract of *B. buteo* could be a reservoir of *van*A-positive enterococci.

Significance and Impact of the Study: First study focused to define the occurrence of *van*A-containing *Enterococcus* strains in *B. buteo*.

IV.4.2. Introduction

Enterococci are part of the normal intestinal flora of humans and animals (Aarestrup et al., 2000); they are also important pathogens responsible for serious human and animal infections (Murray, 1998). With their accumulation of antibiotic resistance, enterococci are recognized as feared nosocomial pathogens that can represent a challenge in treatment of infectious diseases (Murray, 1998). The appearance of vancomycin-resistant enterococci (VRE) in Europe has been associated with the use of avoparcin as growth promoter in food animals (Poeta et al., 2005) until it was forbidden in the European Union in 1997. VRE, particularly *Enterococcus faecium* strains, are frequently resistant to most of the antibiotics that are an effective treatment for vancomycin-susceptible enterococci, which leaves clinicians with limited therapeutic options to treat VRE infections (Murray, 1998). The flow of resistant micro-organisms and resistance genes from livestock and humans to wildlife remains poorly understood even though wild animals may act as reservoirs of resistance that may be amplified and spread across the environment (Blanco et al., 2009). Studies of antimicrobial resistance in the intestinal enterococci of animals and humans have been performed in different countries (Aarestrup et al., 2000; Poeta et al., 2005), but they are scarce in wild animals (Poeta et al., 2005). In this respect, there have been very few studies focusing on the detection of faecal colonization by VRE in wild animals (Poeta et al., 2005). Studies reporting the presence of virulence factors in enterococci of food and animal origin are rare (Eaton & Gasson, 2001), and the occurrence is not documented in faecal enterococci from *Buteo buteo*. This study is the first report of *van*A-containing enterococcal strains in *B. buteo* in Portugal.

The common buzzard (*B. buteo*) is a medium to large bird of prey, with a geographical distribution that covers most of Europe and extends into Asia. As a great opportunist, it is well adapted to a varied diet of pheasant, rabbit, other small mammals, snakes and lizards and can often be seen walking over recently ploughed fields looking for worms and insects. Following this, some studies have demonstrated that its enteric flora depends mainly on prey food (Blanco et al., 2006).

IV.4.3. Material and methods

The presence of VRE was investigated in 33 faecal samples of *B. buteo*. These faecal samples were obtained during September 2007–February 2009, and they were collected individually from each animal in collaboration with Center of Collecting, Welcome and Handling of Wild Animals. This centre is located in the Trás-os-Montes e Alto Douro University and receives injured animals found in their natural environment. None of the animals had previously been fed by humans or received antibiotics. Most of the animals inhabited the Peneda Gêres Natural Park and other natural areas of Portugal subjected to special measures for nature conservation. Faecal samples were diluted and sampled in Slanetz–Bartley agar plates supplemented with 4 mg/L of vancomycin and

incubated for 48 h at 35°C. Colonies with a typical enterococcal morphology (two colonies per sample) were identified to the genus and species level by their cultural characteristics, Gram's strain, catalase test, bile-aesculin reaction and biochemical tests using the API 20Strep system (BioMérieux, La Palme, France). Species identification was confirmed by polymerase chain reaction (PCR), using primers and conditions for the different enterococcal species (Torres et al., 2003). Antibiotic susceptibility was tested for 11 antibiotics of interest in veterinary and human medicine: vancomycin, teicoplanin, ampicillin, streptomycin, gentamicin, kanamycin, chloramphenicol, tetracycline, erythromycin, quinupristin–dalfopristin and ciprofloxacin, by the disc diffusion method (CLSI, 2007). The agar dilution method was also used for susceptibility testing of vancomycin and teicoplanin (CLSI, 2007). *Enterococcus faecalis* strain ATCC29212 and *Staphylococcus aureus* strain ATCC29213 were used as quality controls. Vancomycin resistance genes (*van*A, *van*B, *van*C-1, *van*C-2/3 and *van*D) were tested by PCR in the enterococcal isolates, which showed resistance or reduced susceptibility for glycopeptides (Torres et al., 2003). Resistance genes for other antibiotics, including macrolide [*erm*(A), *erm*(B), *erm*(C)], streptogramine [*vat*(D) and *vat*(E)], tetracycline [*tet*(M), *tet*(L)], aminoglycoside [*aph*(3′)-IIIa, *aac*(6′)-*aph*(2″), *ant*(6)-Ia] and chloramphenicol [*cat*A] resistance genes were tested by PCR in all enterococcal isolates which showed resistance for these antibiotics using previously reported primers and conditions (Torres et al., 2003). A PCR assay was used to demonstrate the presence of the Tn*5397*-like and Tn*916*/Tn*1545*-like transposons (De Leener et al., 2004).

The presence of genes encoding different virulence factors (*gel*E, *fsr*, *esp*, *hyl* and *cyl*$L_L L_S$ABM) was also studied by PCR using previously described primers and conditions (Eaton & Gasson, 2001; Klare et al., 2005; Pillai et al., 2002). Positive and negative controls, obtained from the collection of the University of Trás-os-Montes and Alto Douro (Portugal), were included in all PCR assays. Gelatinase production was detected by inoculating the enterococci on to freshly prepared tryptic soy agar plates containing 1·5% of skim milk (Eaton & Gasson, 2001). The production of haemolysin was determined by streaking bacterial cultures, grown overnight at 37°C in brain heart infusion agar, on columbia agar plates supplemented with 5% horse blood (Eaton & Gasson, 2001). *van*A-containing *E. faecium* isolates were characterized by multilocus sequence typing (MLST). For this purpose, internal 400–600-bp fragments of seven housekeeping genes (*adk*, *atpA*, *ddl*, *gdh*, *gyd*, *purK* and *pstS*) were amplified and sequenced. The sequences obtained were analysed and compared against the http://www.mlst.net database. The combination of the seven obtained alleles for each isolate gave us a specific sequence type (ST) and clonal complex (CC) (Homan et al., 2002).

IV.4.4. Results and discussion

The presence of VRE with an acquired mechanism of resistance was demonstrated in three of the 33 analysed samples (9%), and two isolates per sample were recovered and identified from those positive samples. In many reports, *van*A-containing *E. faecalis* and the *E. faecium* were the most frequently detected species among VRE isolates in poultry (Poeta et al., 2005), although *Enterococcus durans* type *van*A was also found in poultry and sewage (Poeta et al., 2005). In our case, the six recovered *van*A enterococci were identified as *E. faecium* (two isolates) and *E. durans* (four isolates), being interesting for the high proportion of *E. durans* identified in our study. Another important point was the detection of *van*A-containing isolates of different species (*E. faecium* and *E. durans*) in the same sample, something that occurred in two of the three VRE-positive samples. This is an important finding that reflects that the intestinal tract of these animals could contain diverse *van*A-containing microorganisms, and the potential transfer of *van*A genetic determinants among different bacteria in the intestinal tract can not be discarded. The presence of the *van*A determinant in enterococci of three faecal samples is of relevance because this gene is generally located on mobile genetic elements that can be transferred to other microorganisms. The characteristics of these isolates are shown in the Table IV.4.1. *Van*A-containing isolates exhibited a high level of vancomycin [minimal inhibitory concentration (MIC) ≥ 128 mg/L] and teicoplanin resistance (MIC 64 mg/L), and all of them also showed tetracycline and erythromycin resistance. Five of these strains presented both ciprofloxacin and ampicillin resistances, four strains quinupristin–dalfopristin resistance, and one strain kanamycin resistance. Similar results were previously found in isolates of pigs (Aarestrup et al., 2000). The *tet*(M) and/or *tet*(L) genes were found in all tetracycline-resistant *van*A strains, and the Tn*916*/Tn*1545* and/or Tn*5387* transposons were detected in most of them. In a previous study, the *tet*(M) gene was found in 95% of *E. faecium* and *E. faecalis* from humans, pigs and broilers (Aarestrup et al., 2000). All erythromycin-resistant *van*A strains carried the *erm*(B) gene and similar results have been presented by others (Poeta et al., 2005). The *vat*(E) and *vat*(D) genes were detected in four and one quinupristin–dalfopristin resistant *van*A strains, respectively. It is interesting to note the detection in this study of *van*A isolates showing quinupristin/dalfopristin resistance and containing the *vat*(D) or *vat*(E) genes. The *cat*A gene was found in the chloramphenicol-resistant *van*A strain and the kanamycin-resistant *van*A strain harboured the *aph*(3′)-IIIa gene. MLST of the two *van*A-positive *E. faecium* isolates demonstrated the ST273 type in one of them (*E. faecium* But 6B), and the ST5 type in the other one (*E. faecium* But 12A). Both *van*A-containing *E. faecium* isolates showed resistance to ampicillin and ciprofloxacin, and one of them harboured the *hyl* virulence gene. It is important to underline the detection of the ST ST273, a single locus variant of ST18, belonging to the CC CC17, in one of our *van*A-containing *E. faecium* strains, because this CC has been associated with *E. faecium* isolates that are well adapted to hospital environments (Leavis et al., 2006). Very few reports exist in which

virulence factors have been demonstrated in enterococci of food and animal origin (Eaton & Gasson, 2001) and fewer studies have been focused on isolates of wild animals (Poeta et al., 2005). In our study, it is of interest to note that the *E. faecium van*A belonging to ST5 also carried the *cyl*A, *cyl*L_L, *cyl*M, *hyl* and *gel*E virulence genes and showed gelatinase activity.

Enterococci with an intrinsic mechanism of vancomycin resistance were found in nine of the 33 faecal samples (27.3%), and one isolate per sample was kept for further studies. All nine enterococci were identified as *Enterococcus gallinarum* and contained the *vanC*1 gene (Table IV.4.1). The phenotype of resistance and the genes involved in these *E. gallinarum* isolates are shown in Table IV.4.1. It is interesting to note that three of these isolates exhibited a high level of gentamicin resistance and harboured the *aac*(6′)-*aph*(2″) aminoglycoside resistance gene. The *cyl*A, *cyl*B and *cyl*S genes were detected in one *E. gallinarum* isolate and the *esp* and *fsr* genes were not identified among our VRE (Table IV.4.1). None of the *van*A or *van*C1 enterococci showed β-haemolysis when they were grown under aerobic incubation in Columbia agar plates supplemented with 5% horse blood.

Table IV.4.1 | Characteristics of vancomycin-resistant enterococcal strains recovered from buzzards in Portugal

Enterococcus strain	MIC (mg/L)		VAN-genes detected	Resistant phenotype for other antibiotics*	Genes detected by PCR
	VAN	TEC			
E. durans But 6A	>128	64	*van*A	AMP- TET- ERY- CIP- CHL	*tet*(L)+*erm*(B)+*cat*A
E. faecium But 6B	>128	64	*van*A	AMP- TET- ERY- CIP	*tet*(M)+IntTn*916*+*erm*(B)
E. durans But 10A	>128	64	*van*A	AMP- TET- ERY- CIP- QD	*tet*(M)+*tet*(L)+Tn*5397*+*erm*(B)+*vat*(E)
E. durans But 10B	>128	64	*van*A	AMP- KAN- TET- ERY- CIP- QD	*aph*(3´)-IIIa+*tet*(M)+*tet*(L)+Tn*5397*+*erm*(B)+*vat*(E)
E. faecium But 12A	>128	64	*van*A	AMP- TET- ERY- CIP- QD	*tet*(M)+*tet*(L)+IntTn*916*+*erm*(B)+*vat*(E)+*cyl*A+*cyl*L+*cyl*M+*hyl*+*gel*E
E. durans But 12B	>128	64	*van*A	ERY- TET- ERY- QD	*tet*(M)+*tet*(L)+Tn*5397*+*erm*(B)+*vat*(E)+*vat*(D)
E. gallinarum But 7A	8	1	*van*C-1	AMP– TET– ERY– CIP	*tet*(M)+*tet*(L)+IntTn*916*
E. gallinarum But 8A	8	1	*van*C-1	AMP– STR– GEN– TET– ERY	*aac*(6´)-*aph*(2´´)+*tet*(M)+IntTn*916*+*erm*(B)
E. gallinarum But 11A	8	1	*van*C-1	STR– TET– ERY	*ant*(6)-Ia+*tet*(L)
E. gallinarum But 14A	8	1	*van*C-1	STR– TET	*tet*(L)
E. gallinarum But 17A	8	1	*van*C-1	STR– KAN– GEN– TET– ERY– CIP– CHL	*ant*(6)-Ia+*aph*(3´)-IIIa+*aac*(6´)-*aph*(2´´)+*tet*(L)+*tet*(M)+*erm*(B)
E. gallinarum But 21A	8	1	*van*C-1	STR– KAN– GEN– TET– ERY– CHL	*ant*(6)-Ia+*aph*(3')-IIIa+*aac*(6´)-*aph*(2´´)+*tet*(M)+*erm*(B)+*cat*A+*cyl*A+*cyl*B+*cyl*S
E. gallinarum But 24A	8	1	*van*C-1	-	-
E. gallinarum But 27A	8	1	*van*C-1	-	-
E. gallinarum But 29A	8	1	*van*C-1	-	-

MIC, minimal inhibitory concentration

*VAN, vancomycin; TEC, teicoplanin; AMP, ampicillin; STR, high-level streptomycin; KAN, high-level kanamycin; GEN, high-level gentamycin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; QD, quinupristin–dalfopristin; CHL, chloramphenicol

IV.4.5. Conclusion

In summary, a considerable percentage of *B. buteo* carry *van*A-containing isolates of *E. faecium* or *E. durans* species in their faecal content. This work is the first of this type in Portugal, as well as in the Iberia Peninsula, focused on determining the carriage of *van*A-containing enterococcal strains in *B. buteo*. Lower percentages of faecal carriage by *van*A enterococcal strains have previously been detected in other wild animals in Portugal (Poeta et al., 2005, 2006). Many populations of *B. buteo* in northern Europe are migratory and may winter in or pass through Portugal during their migration. However, there are also resident birds that can spread the resistant bacteria and their resistant genes throughout the environment. We cannot rule out the potential for these animals to spread faecal material across the woodland to farm animals or even humans. These modes of transport might be involved in the acquisition and dissemination of antibiotic resistance genes, even in the absence of direct antibiotic pressure. More studies should be carried out to track the evolution of *van*A enterococcal strains across different ecosystems.

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IV.5. Clonal Lineages, antibiotic resistance and virulence factors in vancomycin-resistant enterococci isolated from faecal samples of red foxes (*Vulpes vulpes*)

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IV.5.1. Abstract

Fourteen *van*A-containing enterococcal isolates were detected in seven of 52 faecal samples (13.5%) from free-ranging red foxes in Portugal. Nine of the *van*A-containing isolates were *Enterococcus faecium* and five were *E. durans*. Both sequence types, ST262 and ST273, were identified among *E. faecium* isolates.

IV.5.2. Introduction

Enterococcus spp. are commensals of the gastrointestinal tract of warm-blooded animals and humans. Recently, the incidence of nosocomial enterococcal infections has increased notably (Leavis et al., 2006). The emergence of vancomycin-resistant enterococci (VRE) in Europe has been associated with the use of avoparcin as a feed additive in food-producing animals, until its ban in 1997 by the European Union (European commission, 1997). We evaluated the incidence of antibiotic resistance and virulence factors in VRE isolated from faecal samples of red foxes (*Vulpes vulpes*) in Portugal and determined the circulating genetic lineages among these isolates.

IV.5.3. Material and methods

We collected faecal samples from free-ranging red foxes (one sample per fox) during red-fox hunts, February 2008 to March 2009 in northern Portugal. Samples were diluted and seeded in Slanetz-Bartley agar plates supplemented with 4 mg/L vancomycin and incubated 48h at 35ºC. *Enterococcus* species identification was confirmed by polymerase chain reaction (PCR; (Arias et al., 2006; Dutka-Malen et al., 1995). Antibiotic susceptibility was tested for 11 antibiotics of interest in veterinary and human medicine: vancomycin (30 μg/disk), teicoplanin (30), ampicillin (10), streptomycin (300), gentamicin (120), kanamycin (120), chloramphenicol (30), tetracycline (30), erythromycin (15), quinupristin-dalfopristin (15), and ciprofloxacin (5), by disk diffusion (CLSI, 2007). Minimal inhibitory concentrations of vancomycin and teicoplanin were also determined by agar dilution (CLSI, 2007). Only the category of high-level resistance was considered for streptomycin, gentamicin, and kanamycin. *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used for quality control. Vancomycin resistance genes (*van*A, *van*B, *van*C-1, *van*C-2/3, and *van*D) were tested by PCR in the enterococcal isolates that demonstrated resistance or reduced susceptibility for glycopeptides (Torres et al., 2006). Resistance genes for other antibiotics, including macrolide [*erm*(A), *erm*(B), *erm*(C)], streptogramine [*vat*(D) and *vat*(E)], tetracycline [*tet*(M), *tet*(L)], aminoglycoside [*aph*(3′)-IIIa, *aac*(6′)-*aph*(2″), *ant*(6)-Ia], and chloramphenicol [*cat*A] were tested by PCR (Torres et al., 2006). A PCR assay was performed to demonstrate the presence of the Tn*5397*-like and Tn*916*/Tn*1545*-like transposons (Agersø et al., 2006). Presence of genes encoding different virulence factors (*ace*, *agg*, *cpd*, *gel*E, *esp*, *hyl*, and *cyl*$L_L L_S$ABM) also was identified by PCR (Eaton & Gasson, 2001; Mannu et al., 2003). Positive and negative controls, from the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal, were included in all assays. Gelatinase production was tested by inoculating enterococci onto fresh tryptic soy agar plates containing 1.5% skim milk (Gilmore et al., 2002). Production of hemolysin was determined by streaking bacterial cultures on Columbia agar supplemented with 5% horse blood (Semedo et al., 2003). *Enterococcus faecium van*A isolates were characterized by multilocus

sequence typing. For this purpose, internal 400- to 600-bp fragments of seven housekeeping genes (*adk*, *atp*A, *ddl*, *gdh*, *gyd*, *pur*K, and *pst*S) were amplified and sequenced. Sequences were analyzed and compared with the database www.mlst.net. The combination of the seven alleles for each isolate, gave us a specific sequence type (ST) and clonal complex (Homan et al., 2002).

IV.5.4. Results and discussion

Two vancomycin-resistant enterococcal isolates from each positive faecal sample were kept for further study. Vancomicin-resistant enterococci with an acquired mechanism of glicopeptide resistance (*van*A genotype) were detected in seven of the 52 faecal samples (13.5%), and nine of the *van*A-containing isolates were identified as *E. faecium* (from five samples) and five isolates as *E. durans* (from three samples; Table IV.5.1). All 14 *van*A-containing isolates exhibited erythromycin resistance and harbored the *erm*(B) gene. The association of *tet*(M) and *tet*(L) genes was found in seven tetracycline-resistant *van*A isolates, and the *tet*(M) or *tet*(L) genes in three additional tetracycline-resistant isolates. The Tn*916* transposon was detected in five *tet*(M)-positive *van*A isolates (Table IV.5.1). In a previous study, *tet*(M) was associated predominantly with Tn*916*/Tn*1545*-like transposons in *E. faecium* from pigs (*Sus scrofa*) and humans, as well as in E. faecalis from humans, pigs, and broilers (*Gallus gallus*; (Agersø et al., 2006)).

The *aph*(3')-IIIa gene was demonstrated in the three isolates that presented high-level kanamycin resistance and the *aac*(6')-*aph*(2") gene in all the gentamicin-resistant *van*A strains. The *vat*(E)/*vat*(D), *ant*(6)-*Ia*, and *cat*A genes were not found among our quinupristin-dalfopristin, streptomycin, and chloramphenicol-resistant *van*A isolates, respectively (Table IV.5.1).

Multilocus sequence typing of the nine *van*A-positive *E. faecium* isolates demonstrated the ST273 sequence type in six and the ST262 type in three. Both ST262 and ST273 are single-locus variants of ST18 and are included in the high-risk clonal complex CC17, frequently associated with hospital environments; in addition, isolates of these STs frequently show ciprofloxacin and ampicillin resistance (Leavis et al., 2006), as is the case for most of our *van*A-containing *E. faecium* isolates.

Enterococci with an intrinsic mechanism of vancomycin resistance (*van*C-1 gene intrinsic of *E. gallinarum* species) were found in four of the 52 red-fox faecal samples (7.7%), and eight isolates were recovered (two per positive sample). The characteristics of these *E. gallinarum* isolates are shown in Table IV.5.1. The epidemiologic interest of this type of intrinsic resistance is low because it cannot be transferred horizontally to other microorganisms.

None of the VRE isolates either with *van*A or *van*C genotype exhibited β-hemolysis, but twelve of the 14 *van*A-containing isolates and six of the eight *van*C-1 isolates exhibited gelatinase activity. The *gel*E gene was also detected in one *van*A-containing *E. faecium* gelatinase-negative isolate (HR 18B isolate). Correspondingly, this gene was also detected in *E. faecalis* and *E. durans* gelatinase-negative isolates recovered from poultry (Poeta et al., 2006).

Table IV.5.1 | Characteristics of vancomycin-resistant enterococcal isolates recovered from *Vulpes vulpes* in Portugal

Enterococcus strain	MIC (mg/L)[a]		VAN genes detected	ST (CC)	Resistant phenotype for other antibiotics[a]	Other resistance genes detected	Genes encoding virulence factors
	VAN	TEC					
E. faecium HR 8A	>128	64	*van*A	ST273 (CC17)	AMP-ERY-CIP	*erm*(B)	*hyl*, *cyl*L_L, *gel*E
E. faecium HR 8B	>128	64	*van*A	ST273 (CC17)	AMP-ERY-CIP	*erm*(B)	*gel*E
E. faecium HR 10A	>128	64	*van*A	ST262 (CC17)	AMP-GEN-KAN-TET-ERY-CIP	*aac*(6′)-*aph*(2′′)+*aph*(3′)-IIIa+*tet*(L)+*erm*(B)	*agg*, *gel*E
E. faecium HR 10B	>128	64	*van*A	ST273 (CC17)	AMP-KAN-ERY-CIP	*aph*(3′)-IIIa+*erm*(B)	*gel*E
E. durans HR 13A	>128	64	*van*A		AMP-TET-ERY-CIP	*tet*(M)+*tet*(L)+Tn*916*+*erm*(B)	*agg*, *gel*E
E. durans HR 13B	>128	64	*van*A		TET-ERY	*tet*(M)+*tet*(L)+Tn*916*+*erm*(B)	*agg*, *gel*E
E. durans HR 14A	>128	64	*van*A		AMP-TET-ERY-CIP	*tet*(M)+*tet*(L)+Tn*916*+*erm*(B)	*agg*, *gel*E
E. faecium HR 14B	>128	64	*van*A	ST273 (CC17)	AMP-ERY-CIP	*erm*(B)	*gel*E
E. faecium HR 16A	>128	64	*van*A	ST273 (CC17)	AMP-TET-ERY-CIP	*tet*(M)+Tn*916*+*erm*(B)	*gel*E
E. faecium HR 16B	>128	64	*van*A	ST273 (CC17)	AMP-TET-ERY	*tet*(M)+*erm*(B)	*gel*E
E. faecium HR 18A	>128	64	*van*A	ST262 (CC17)	AMP-STR-KAN-TET-ERY-CIP	*aph*(3′)-IIIa+*tet*(M)+*tet*(L)+*erm*(B)	*gel*E
E. faecium HR 18B	>128	64	*van*A	ST262 (CC17)	AMP-GEN-TET-ERY-CIP-QD-CHL	*aac*(6′)-aph(2′′)+*tet*(M)+*tet*(L)+Tn*916*+*erm*(B)	*gel*E, *cyl*L_L
E. durans HR 33A	>128	64	*van*A		AMP-GEN-TET-ERY-CIP	*aac*(6′)-aph(2′′)+*tet*(M)+*tet*(L)+*erm*(B)	-
E. durans HR 33B	>128	64	*van*A		AMP-ERY-CIP-QD	*tet*(M)+*tet*(L)+*erm*(B)	-
E. gallinarum HR 2A	8	1	*van*C-1		STR-TET-ERY-CIP-QD-CHL	*tet*(M)+*tet*(L)+*erm*(B)	-
E. gallinarum HR 2B	8	1	*van*C-1		STR-KAN-TET-ERY-QD	*aph*(3′)-IIIa+*tet*(M)+*tet*(L)+*erm*(B)	*esp*, *gel*E
E. gallinarum HR 6A	8	1	*van*C-1		KAN-TET-CIP-QD	*aph*(3′)-IIIa+*tet*(M)+Tn*916*	*esp*, *gel*E
E. gallinarum HR 6B	8	1	*van*C-1		KAN-TET-CIP-QD	*aph*(3′)-IIIa+*tet*(M)	*esp*, *gel*E
E. gallinarum HR 38A	8	1	*van*C-1		STR-KAN-TET-QD	*aph*(3′)-IIIa+*tet*(M)+Tn*5397*+*vat*(D)	*gel*E
E. gallinarum HR 38B	8	1	*van*C-1		TET-QD	*tet*(M)+*tet*(L)+Tn*5397*	*gel*E
E. gallinarum HR 48A	8	1	*van*C-1		TET-CIP	*tet*(M)+*tet*(L)	*cyl*L_L
E. gallinarum HR 48B	8	1	*van*C-1		ERY	*erm*(B)	*cpd*

[a]VAN, vancomycin; TEC, teicoplanin; AMP, ampicillin; STR, high-level streptomycin; KAN, high-level kanamycin; GEN, high-level gentamycin; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin; QD, quinupristin-dalfopristin; CHL, chloramphenicol

IV.5.5. Conclusion

To our knowledge, this is the first report of *van*A-containing enterococcal isolates in red foxes, and a high percentage of animals carried this type of acquired resistance mechanism, usually located on transferable plasmids. Studies of wild Buzzards (*Buteo buteo*) or domestic pigs in Portugal showed similar percentages of *van*A-containing enterococci (9–13%; (Gonçalves et al., 2010; Radhouani et al., 2010). It seems that acquired vancomycin resistance in enterococci is frequent in nonhuman ecosystems. According to EARS-Net (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx), Portugal is one of the European countries that report higher percentages of vancomycin resistance among invasive *E. faecium* and *E. faecalis* isolates. Dissemination of these resistant microorganisms in different ecosystems is possible. Surveillance should be continued to characterize the evolution of VRE in different ecosystems, including in wild animals of other species and countries.

IV.5.6. Acknowledgements

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PART III

PROTEOMICS APPROACH

CHAPTER V

CHAPTER VI

CHAPTER V

PROTEOMIC CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT *E. COLI* ISOLATES IN WILD ANIMALS

CHAPTER V

V.1. Pinto L, Poeta P, Radhouani H, et al. (2011) **Proteomic study in an *Escherichia coli* strain from seagulls of the Berlengas Natural Reserve of Portugal.**

V.2. Radhouani H, Nunes-Miranda JD, Carreira R, et al. (2013) **Proteomic changes in an extended-spectrum beta-lactamase-producing *Escherichia coli* strain under cefotaxime selection.**

V.1. Proteomic study in an *Escherichia coli* strain from seagulls of the Berlengas Natural Reserve of Portugal

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V.1.1. Abstract

The increasing bacterial resistance among common pathogen is threatening the effectiveness of several antibiotics. This represents a serious public health problem as such bacterial strains have already been detected in domestic, wildlife animals and humans. Using *Escherichia coli* as a model organism, we applied a proteomic approach to the topic of antimicrobial resistance. In order to identify and characterize the proteome of extended-spectrum β-lactamase (ESBL) type TEM-52 producing-*Escherichia coli* strain of a faecal sample taken from Yellow-legged seagulls (*Larus cachinnans*) a bidimensional electrophoresis (2-DE) technique with an isoelectric focusing followed by a SDS-PAGE was used. Eighty-seven individualized protein spots were identified. All were suitable for peptide mass fingerprinting by a mass spectrometric technique (MALDI/TOF MS). Their identification was carried out by searching appropriate bioinformatic databases. All proteins were related to *E. coli* strains. Detection of proteins related to several *E. coli* strains linked with virulent and enterohaemorrhagic consequences in ESBL producing-*E. coli* isolates of seagull samples raises the question of how such similarities arise bearing in mind these remarkably different microbial ecosystems.

V.1.2. Introduction

Proteome analysis of innumerable biological organisms represents a major challenge for the post-genomic era and constitutes an abundant source of biological information (Patterson & Aebersold, 2003). In analogy to the genome, the proteome is used to describe a set of proteins expressed by a certain organism, under defined conditions, serving as a unique and informative readout of both its phenotypic state, which results from cell responses to physiological and environmental perturbations, and genomic information reflected in the amino acid sequences of expressed proteins. The main concern of proteomic resides, then, in the identification of proteins involved in particular cellular processes or presenting altered expression profiles as a consequence of different physiological conditions (Panisko et al., 2002). Currently, increasing rates of bacterial resistance exist among common pathogens that are threatening the effectiveness of the most powerful antimicrobials. The emergence and spread of antibiotic-resistant Gram-negative pathogens, such as *Escherichia coli* can lead to serious public health issues for humans and animals (Fish & Ohlinger, 2006). *E. coli*, a very well characterized prokaryote, has served as a model organism for several biological and biotechnological studies increasingly so since the completion of the *E. coli* genome-sequencing project (Han & Lee, 2006). The availability of complete genome sequence databases therefore facilitates the proteomic analysis of *E. coli* using MS (Mass Spectrometry). The most common resistance mechanism in *E. coli* and other Enterobacteriaceae is through the production of β-lactamases–enzymes which inactivate certain β- lactam antibiotics (Jacoby & Munoz-Price, 2005). Extended-spectrum β-lactamases (ESBLs) are considered a major mechanism of resistance to β-lactam antibiotics that include broad-spectrum cephalosporins and aztreonam (Bush, 2001; Mendonca et al., 2007). In fact, nowadays, ESBL-containing bacteria represent a major threat to the human community and for hospital patients causing several outbreaks and becoming endemic in many hospitals around the world (Perez et al., 2007). The use of antibiotics in food-producing animals could lead to selection of antibiotic-resistant bacteria that can be transferred to humans through the consumption or handling of foods of animal origin. In recent studies it was demonstrated that antimicrobial resistance in foodborne bacteria might result in prolonged illness and elevated rates of bacteremia, hospitalization and death (Tollefson, 2004). In fact, pathogenic strains of *E. coli* can cause sepsis and infections of the nervous system, digestive and urinary tracts (Gustafsson et al., 2001) and their presence in animals in contact with humans becomes a matter of great concern. The traditional method of measuring protein expression is by two-dimensional gel electrophoresis (2-DE) which, combined with the mass spectrometry (MS) of protein spots, makes up one of the most prevalent techniques used in proteomics, capable of generating a portrait of the global protein expression in a given sample (Celis & Gromov, 1999; Dumas et al., 2009; Yarmush & Jayaraman, 2002). Two-dimensional gel electrophoresis is a long established technology by which proteins are separated according to their isoelectric point (pI) and

their molecular weight (Mr) (O'Farrell, 1975). The high-throughput identification of proteins excised from 2-DE gels was demonstrated by Shevchenko and colleagues (1996), who used this procedure to identify proteins expressed in yeast, following the resolution of a cell lysate by 2-D PAGE (Shevchenko et al., 1996). MS-based proteomics normally involves large-scale identification, quantification, and characterization of proteins at various levels resulting in an important tool for the analysis of biological systems and the exploration of complex protein functions and interactions. The most common method of protein identification is the analysis of peptide masses resulting from enzymatic digestion (e.g. by trypsin) of proteins resolved by and excised from 2-DE, by using MALDI-TOF MS (Shevchenko et al., 1996). In order to make sequence data derived from several bacterial genomes accessible via public databases, proteome data are characterized by diverse data types and are stored in proprietary databases located worldwide (Hiscock & Upton, 2000; Oh et al., 1999), becoming a reliable source of information for proteomic studies. The evaluation of protein profiles in response to various stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance, could represent a valid and integrating approach for the development of new therapeutic strategies. In the current study, a proteomic evaluation of an *E. coli* isolate (GV5), recovered from a faecal sample of a Yellow-legged seagull (*Larus cachinnans*) from Berlengas Natural Reserve of Portugal and carrying an ESBL TEM-52, was performed by 2-DE and subsequent protein identification by MALDI-TOF MS.

V.1.3. Material and methods

V.1.3.1. Cell culture and purification of E. coli

E. coli strains were obtained from faecal samples of Yellow legged seagulls (Figure V.1.1), randomly recovered in the beaches of Berlengas Islands National Reserve of Portugal, located at the coast of Peniche (GPS coordinates: N 39. 24' 51,77", W 9. 30' 33,67"). They were previously characterized for antibiotic resistant genes (Poeta et al., 2008). One of these *E. coli* strains, GV5, was included in this study. This strain was proven to be a β- lactamase TEM-52 producer (Vinué et al., 2008).

Figure V.1.1 | Seagulls (*Larus cachinnans*) in Berlengas archipelago natural reserve

V.1.3.2. Protein extraction

Exponentially growing cells (15 mL) were harvested by centrifugation (3 min, 10,000xg, 4ºC) and re-suspended in PBS (4 mL) at room temperature, followed by a second centrifugation and re-suspention with SDS+Tris solution (0.2 mL) (Celis & Gromov, 1999). Cell disruption was performed by sonication (3×10 s, 4ºC at 100 W); cell debris was removed by centrifugation (14,000×g, 30 min at 4ºC). The protein concentration was assayed using a 2D Quant kit (GE Healthcare).

V.1.3.3. One-dimensional electrophoresis and staining

One-dimensional electrophoresis was conducted with SDS polyacrylamide gels (T=12.52%, C=0.97%) in a Hoefer™ SE 600 Ruby (Amersham Biosciences) unit, following Laemmli (Laemmli, 1970) with some specific modifications (Igrejas, 2000). Gels were stained during 24 hours in Coomasse Brilliant Blue R-250 and washed in water overnight. It was then fixed in trichloroacetic acid (6%) for four hours and in glycerol (5%) for two hours (Görg et al., 2000).

V.1.3.4. Two-dimensional electrophoresis and proteome analysis

2-DE was performed according to the principles of O'Farrell (1975) but with IPG (Immobiline™ pH Gradient) technology (Laemmli, 1970). Protein samples of *E. coli* (GV5) were used in parallel with samples of *E. coli* C583 and C580. For IEF, precast IPG strips with linear gradient of pH 3-10 were passively rehydrated overnight (12 to 16 hours) in a reswelling tray with rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte IPG buffer pH 3-10) at room temperature. IPG strips were covered with DryStrip Cover Fluid (Plus One, Amersham Biosciences). Lyses buffer [9.5 M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3-10) and 10 mM Pefabloc. proteinase inhibitor] was added to the *E. coli* isolates (1:1). Samples containing a total of 100 µg of protein were loaded into 13 cm IPG strips (pH 3–10 NL, Amersham Biosciences, UK) (Görg et al., 2000). The sample solution was then applied to the previously rehydrated IPG strips pH3-10 by cup loading and then proteins were focused sequentially at 500 V for 1 h, gradient at 1000 V for 8 h, gradient at 8000 V for 3 h, and finally 8000 V during 1 h on an Ettan™ IPGPhor II™ (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed according to Görg (Görg et al., 2007) and the GE Healthcare protocol for IPG strips pH 3-10 of 13 cm, in order to obtain the optimized running conditions, resulting in a final 13 hours run. Focused IPG strips were then stored at -80ºC in plastic bags. Before running the second dimension, strips were equilibrated twice for 15 minutes in equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer (pH 8.8)). In the first equilibration 1% DTT was added to the original equilibration buffer and to the second, 4% iodoacetamide. Bromophenol blue was added to both solutions. The equilibrated IPG strips were gently rinsed with

SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied to 12.52% polyacrylamide gels in a Hoefer™ SE 600 Ruby (Amersham Biosciences) unit. Some modifications were introduced in the SDS-PAGE technique previously reported by Laemmli (1970) that allowed its resolution to be increased, with proper insertion of the IPG strips in the stacking gel (Igrejas, 2000; Laemmli, 1970). After SDS-PAGE, the 2-DE gels were fixed in 40% methanol/10% acetic acid for one hour and afterwards stained overnight in Coomassie Brilliant Blue G-250 (Görg et al., 2004). Coomassie-stained gels were scanned on a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA), and the resulting digitized images were analyzed using Image Master 5.0 software (Amersham Biosciences; GE Healthcare).

V.1.3.5. Protein identification by MALDI-TOF/TOF

To increase experimental efficacy, four separate gels were analyzed originally representing three independent *E. coli* protein samples that were previously pooled together and compared. Spots of expression in all gels were manually excised from the gels and analyzed using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). The gel pieces were washed three times with 25 mM ammonium bicarbonate/50% ACN (acetonitrile), once with ACN and dried in a SpeedVac (Thermo Savant). 25 mL of 10 mg/mL sequence grade modified porcine trypsin (Promega) in 25 mM ammonium bicabornate was added to the dried gel pieces and the samples were incubated overnight at 37ºC. Extraction of tryptic peptides was performed by addition of 10% of formic acid (FA)/50% ACN followed by three-fold lyophilisation in a SpeedVac (Thermo Savant). Tryptic peptides were re-suspended in 10 mL of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/0.1% formic acid. Aliquots of samples (0.5 μL) were spotted onto the MALDI sample target plate. Peptide mass spectra were obtained on a MALDITOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Mass spectra were internally calibrated with autodigest peaks of trypsin (MH+: 842.5, 2211.42 Da) allowing a mass accuracy of better than 25 ppm.

V.1.3.6. Database search

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v 2.1.04, Matrix Science, London, UK) for searching the peptide mass fingerprints and MS/MS data. Swiss-Prot nonredundant protein sequence

database (Release 57 of March 2009, 428650 entries) was used for all searches under *E. coli*. The database search parameters were as follows: carbamidomethylation and propionamide of cysteine (+71Da) as a variable modification as well as oxidation of methionine (+16Da), and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 25 ppm and fragment ion mass tolerance was 0.3 Da. Positive identifications were accepted up to 95% of confidence level. Protein identifications were considered as reliable when the MASCOT score was > 70 (MASCOT score was calculated as $-10\times\log P$, where P is the probability that the observed match is a random event.). This is the lowest score indicated by the program as significant ($P< 0.05$) and indicated by the probability of incorrect protein identification.

V.1.4. Results and Discussion

E. coli strains included in the study were previously characterized for virulence and antibiotic resistance. The seagull's ESBL-producing *E. coli* strain GV5 presented resistance to ampicillin, cefotaxime, ceftazidime, naladixic acid, ciprofloxacine, tetracycline, streptomycin, trimethoprim/sulfametoxazole, and chloramphenicol and contained the following resistance genes: $bla_{\text{TEM-52}}$ (ESBL gene encoding TEM-52) *cml*A (chloramphenicol-resistance), *tet*(A) (tetracycline-resistance), *sul*1 and *sul*3 (sulfametoxazole-resistance). The *intI*1gene encoding the integrase of class I integrons was also identified in strain GV5, as well as the gene cassettes *dfr*A1+*aad*A1 included in their variable region. *E. coli* GV5 was classified into the phylogenetic group D, mostly associated with virulent extraintestinal isolates (Poeta et al., 2008). In sample GV5 a total of 87 protein spots were collected and individualized in eppendorfs for their analysis using MALDI-TOF mass spectrometry. The protein spots were identified and characterized (Table S2) by correlation with bioinformatics databases (http://www.ncbi.nlm.nih.gov/). From a total of 87 protein spots, nine proteins were detected as related to *E. coli* serotype O6 and another six proteins linked with *E. coli* serotype O157:H7 (Figure V.1.2, A and B respectively; see also Table S2). Among the proteins identified as linked to serotype O6, it is important to highlight the presence of proteins related to environmental stress conditions: the curved DNA-binding protein (spot 18), the chaperone protein (spot 35) and the GrpE protein (spot 45) (Blattner et al., 1997; Perna et al., 2001; Welch et al., 2002) are responsible for the capacity of the bacteria to resist external damage (heat, osmotic and other environmental stress causes). Beta-lactamase TEM (P62593, spot 39) and dihydrofolate reductase type XV (P78218, spots 8) were related to antibiotic resistance. The trigger factor protein (or tig), on other hand, is involved in the protein exportation whereas the other proteins related to serotype O6 presented functions in important metabolic pathways essential for the bacteria. The proteins found to be related to serotype O157:H7 presented various functions, as kdsA protein (spot 60) with an important part in the external membrane and lipopolysaccharide biogenesis (Perna et al., 2001).

The curved DNA-binding protein previously identified for the O6 serotype, was also found in the O157:H7 serotype (spot 18). The *E. coli* serotype O6 represents a heterogeneous group of bacteria, which differ in the genotypic presence as well as in the phenotypic expression of virulence factors, being already detected in humans, dogs and cats (Johnson et al., 2008). On the other hand, the serotype O157:H7 is an enterohemorrhagic strain that belongs to the verotoxin-producing *E. coli* (VTEC). VTEC have emerged as food-borne pathogens related to gastroenteritis that may be complicated by hemorrhagic colitis or by the hemolytic uremic syndrome, the main cause of acute renal failure in children (Blanco et al., 2003). The transmission of these foodborne pathogens occurs through consumption of undercooked meat, unpasteurized dairy products, vegetables, water contaminated by ruminant feces as these animals can carry *E. coli* O157:H7 transiently and sporadically and pass the bacteria in their feces (Blanco et al., 2003; Cray & Moon, 1995; Wallace, 1999). The proximity of the Berlengas Natural Reserve to the Continental Portuguese coastline is an important factor in considering the possible contact between seagulls and potential disease transmitters. In fact, vero cytotoxin- producing *E. coli* O157 has already been detected in the past in faecal samples from wild-birds (Foster et al., 2006) namely from gulls (Wallace et al., 1997), determining probably a transmission pattern. 2-DE proteomics correlated with bioinformatic databases has already been used in analyzing and characterizing bacterial proteins, as in *E. coli* where a large number of proteins are already completely identified (Han & Lee, 2006; Lopez-Campistrous et al., 2005). In our study a great number of proteins which, were related to several functions within the cell metabolism were found, like β-lactamase TEM precursor proteins (blaT) capable of producing β-lactamases (Figure V.1.3). The blaT proteins are prevalent in enterobacteriaceae hydrolyzing the β-lactam bond of susceptible β-lactam antibiotics like penicillins and cephalosporins (Brun et al., 1994; Mabilat et al., 1992). The presence of three protein spots identified as blaT (spots 39, 40 and 79; see Table S2) confirms that our sample possesses resistance to β-lactam antibiotics. Other proteins relevant to various important and essential functions in the bacteria were found. Flagellin or FliC (spot 14) is responsible for the formation of bacterial flagella (Blattner et al., 1997; Hayashi et al., 2006); chaperone protein or DnaK (spots 1, 49 and 62), involved in the chromosomal DNA replication (Bardwell & Craig, 1984; Bardwell et al., 1986; Pellecchia et al., 2000); while L-asparaginase II or AspG2 (spot 53), is an important therapeutic enzyme for the treatment of leukemia (Blattner et al., 1997; Derst et al., 1992). In the case of enterohemorrhagic *E. coli* strain, four related proteins were found in *E. coli* isolates GV5 (Putative flavoprotein, Serine hydroxymethyltransferase protein and Curved-DNA binding protein). Also noticed was the detection of proteins Malate dehydrogenase and GrpE protein linked with *E. coli* enterohemorrhagic strain O157:H7 in *E. coli* virulent strain O6 in the seagull isolate. The presence of proteins associated with such *E. coli* strains alongside proteins related to commensal strains in faecal samples of wildlife animals and their resistance to antimicrobial drugs represents a public health concern. The idea of antimicrobial resistance in the same extended-spectrum

β-lactamase *E. coli* of very different ecosystems as microbial fauna of wildlife animals and humans with repressed imunosystems is therefore a new concern in the already problematic question of antibiotic resistance and possible transmission.

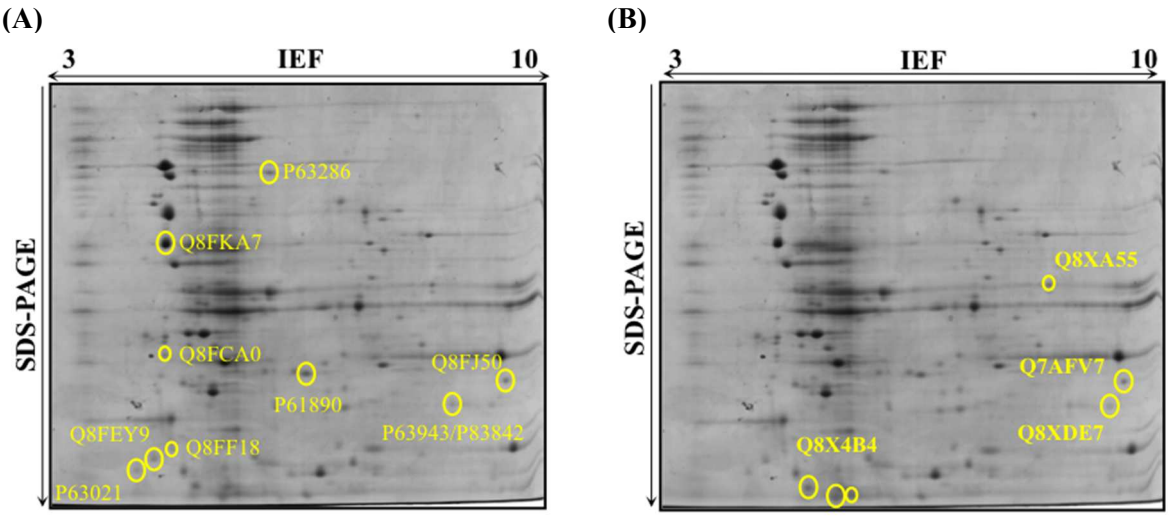

Figure V.1.2 | 2-DE gel image of *E. coli* sample GV5

(A) Accession numbers of proteins related to *E. coli* serotype O6; (B) Accession numbers of proteins related to *E. coli* serotype O157:H7.

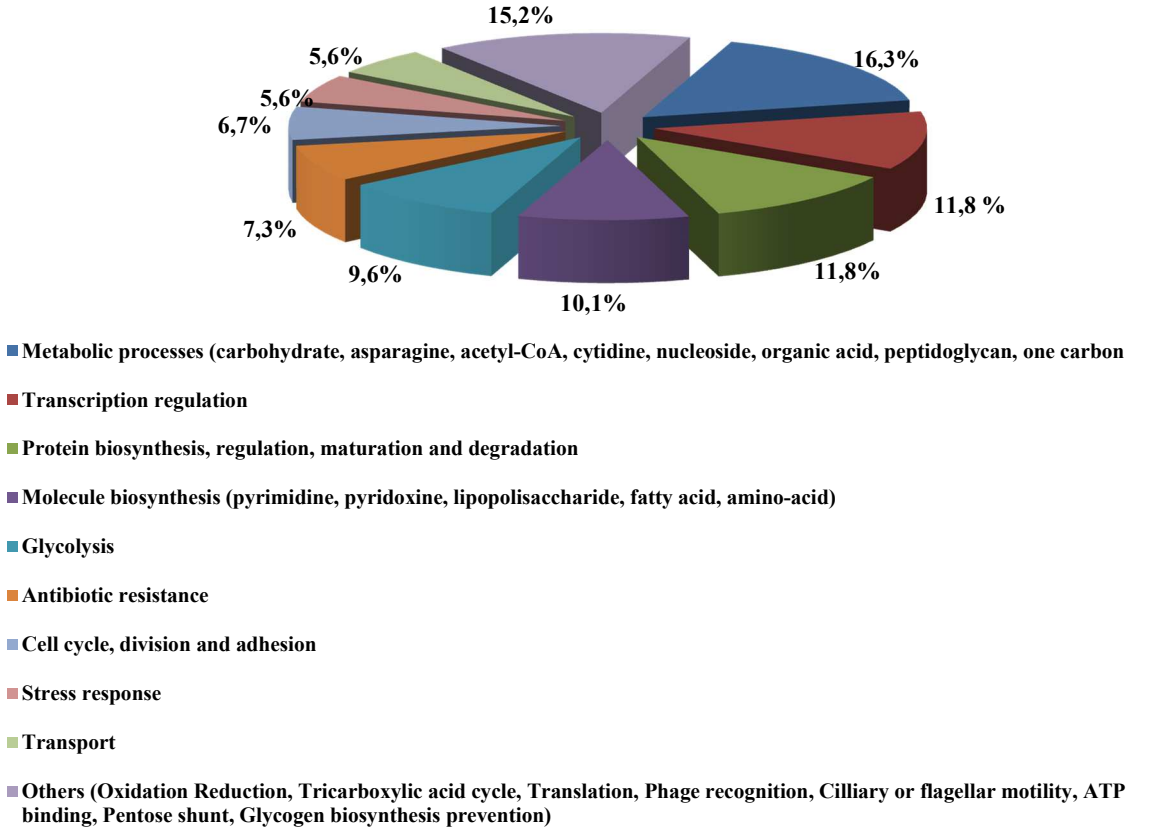

Figure V.1.3 | Distribution of the biological processes related to the protein spots found in the 2-DE gels of the *E. coli* GV5

V.1.5. Concluding remarks

In this study, the elaboration of a 2-DE electrophoresis gel of an extended-spectrum β-lactamase *E. coli* strain with phenotypic and genotypic profiles indicating antimicrobial resistance allowed us to identify and characterize the proteins present. The proteome patterns obtained reveal proteins previously identified in the virulent strain *E. coli* O6 and enterohemorrhagic strain *E. coli* O157:H7. The detection of proteins related to these strains in samples of extended-spectrum β-lactamase-producing *E. coli* isolates became possible through the proteomic approach and 2-DE combined with mass spectrometry. Considering this work, it is possible to elucidate gene expression of multiresistant bacteria strains isolated from different wild ecosystems. In the future it will be important to evaluate this expression under different forms of stress. These proteins should be tested under stress conditions, for example under antibiotic pressure, in order to determine the changes in protein expression, and to test potential targets for designing new drugs to inhibit the growth of the antibiotic-resistant bacteria.

V.1.6. Supplementary material

Supplementary material (Table S2) regarding this manuscript is available at JIOMICS online (http://www.jiomics.com/index.php/jio/rt/suppFiles/20/0).

V.1.7. References

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V.2. Proteomic changes in an extended-spectrum beta-lactamase-producing *Escherichia coli* strain under cefotaxime selection

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V.2.1. Abstract

Proteomics can be used to study the metabolic pathways and mechanisms involved in antimicrobial resistance. The aim of this comparative proteomic study was to establish the overall changes in the proteome of a naturally occurring ESBL-producing *E. coli* strain (C5478) stressed with its minimal inhibitory concentration (2 μg/mL) of cefotaxime, compared to the proteome of the same strain without antimicrobial stress, by using 2-D gel electrophoresis (2-DE) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The comparative proteomic analysis revealed that the abundance of numerous protein species changed in the strain stressed by CTX compared to the non-stressed wild-type strain. A total of 188 spots were excised from the 2-DE gel of the wild-type strain, 112 of which were successfully identified by MALDI-TOF MS, representing 110 different proteins. Concerning the 2-DE gel of the CTX-stressed bacteria, 171 spots were excised and 156 were identified, representing 143 different proteins. The proteins identified in both strains were categorized according to their biological functions. These proteins were involved in metabolism, protein synthesis, cell division, stress responses, and antimicrobial resistance, among others. These findings will be helpful to further understand not only the antimicrobial resistance mechanisms, but also the role of wild animals as reservoirs in spreading antimicrobial-resistant bacteria into the environment.

V.2.2. Introduction

Cefotaxime is one of the most widely used parenteral third generation cephalosporins in medicine (Tracz et al., 2005). However, shortly after the introduction of the first β-lactam antibiotics, some bacteria were found to be resistant, comprising the long-term usefulness of these drugs (Carter et al., 2005). A common resistance mechanism is the expression of β-lactamase enzymes by bacteria, which inactivate the antibiotics by cleaving the β-lactam core. Resistant extended-spectrum β-lactamases (ESBL)-producing bacteria are thus a major clinical and public health concern in various countries, including Portugal, as they are commonly implicated in human infections (Paterson & Bonomo, 2005).

The appearance of multiresistant bacterial strains in humans or animals in clinical situations can also lead to contamination of the environment (Grobbel et al., 2007). The production of ESBLs by *Enterobacteriaceae*, and especially *Escherichia coli*, has been repeatedly described in apparently healthy animals in the last few years (Ewers et al., 2011). Wildlife is not usually exposed to antimicrobial agents in clinical use but may acquire antimicrobial-resistant bacteria through contact with humans, domesticated animals and the environment. Water polluted with faeces is probably the most significant route of contamination. Monitoring the prevalence of resistance in indicator bacteria such as *E. coli* in wild animals could show whether wildlife serves as an environmental reservoir or "melting pot" of bacterial resistance.

Proteomics techniques can be used to quantitatively compare the expression of protein in different conditions to obtain a better understanding of the cellular processes affecting their expression and/or in which they are involved. Mostly two-dimensional electrophoresis (2-DE) is used for extensive protein separation before identification. Mass spectrometry (MS) in combination with various protein resolution methods and bioinformatics tools is used routinely in proteomics research (Jungblut et al., 2008). For example, 2-DE and MALDI-TOF MS have been used several times to identify antimicrobial-related proteins in *E. coli* strains (Pinto et al., 2011a, 2011b; Radhouani et al., 2012b; Roncada et al., 2009).

The scientific community is developing several tools in order to address the problem of antimicrobial resistance (Reinhart & Hartog, 2010; Werner et al., 2008). Proteomics research offers major opportunities to characterize bacterial pathogens, elucidate disease mechanisms and to identify new diagnostic markers and therapeutic targets (Josic & Kovac, 2008). Comparative proteomic analyses of different bacterial species have shown that a subproteome related to resistance mechanisms changes when some strains are challenged with antimicrobials, e.g., in *E. coli* isolates treated with piperacillin/tazobactam (dos Santos et al., 2010), nalidixic acid (Lin et al., 2008), tetracycline (Xu et al., 2006; Zhang et al., 2008), streptomycin (Li et al., 2008), chloramphenicol (Li et al., 2007) and ampicillin (Xu et al., 2006); in *Salmonella* treated with fluoroquinolones (Coldham et al., 2006); in *Stenotrophomonas maltophilia* treated with imipenem (Liu et al., 2012); and in *Acinetobacter baumannii* treated with tetracycline and imipenem (Yun et al., 2011).

To better understand the universal pathways that form barriers to antimicrobial agents, the aim of this comparative proteomic study was to establish the overall changes between a wild-type strain (ESBL-producing *E. coli* strain C5478) and the same strain stressed with cefotaxime.

V.2.3. Material and methods

V.2.3.1. Isolation of bacteria from fox faeces

The *E. coli* strain C5478, referred to as the wild-type strain, was characterized in a previous study (Radhouani et al., 2012a). Red fox faecal samples were collected from February-2008 to March-2009 in North of Portugal (where these animals live in the wild) during hunts of red foxes. This kind of hunting is organized all of the years during a short period of time having like main objective the ecological control of the animal population and is supervised by the Agriculture, Rural development and Fishery Ministry of Portugal under the Decree-Law no. 202/2004. The animals were obtained in collaboration with National Corporation of Forest Rangers. As far as we know, none of the animals had received antimicrobials. Faecal samples were seeded into Levine agar supplemented with CTX (2 μg/mL).

V.2.3.2. Genetic characterization of E. coli C5478

C5478 showed resistance to ampicillin, amoxicillin + clavulanic acid, CTX, ceftazidime, aztreonam, tetracycline, sulfamethoxazole/trimethoprim, streptomycin, nalidixic acid, ciprofloxacin and chloramphenicol. The β-lactamase genes detected in C5478 are $bla_{\text{SHV-12}} + bla_{\text{TEM-1b}}$. C5478 also harbours the genes *tet*(A) and *sul*2 and contained the non-classical class 1 integron (*intI*1-*dfr*A12-*orf*F-*aad*A2-*cml*A1-*aad*A1-*qacH*-IS*440*-*sul*3) with the PcH1 promoter. The presence of two amino acid changes in GyrA (S83L and D87G) and one in ParC (S80I) were responsible for the ciprofloxacin and nalidixic acid resistance phenotypes detected in C5478. This isolate was typed as sequence type ST1086, phylogroup A and carried the *fim*A virulence gene (Radhouani et al., 2012a).

V.2.3.3. Culture conditions and total protein extraction

The "stressed strain", was obtained by culturing the "wild-type strain" (ESBL-producing *E. coli* strain C5478) into brain heart infusion (BHI) broth containing 2 μg/mL CTX (Oxoid, Cambridge, UK). For this study, *Escherichia coli* strain K-12 was used as the negative control. In the post-exponential phase (when the optical density of the culture OD_{540nm} = 6 corresponding to 2–3 × 10^9 cells/mL), the cells were pelleted at 10,000 rpm at 4ºC for 3 min. The pellet was resuspended in an equal volume of pre-warmed phosphate-buffered saline (PBS) pH 7.4 (Görg et al., 2004). After a second centrifugation, the pellet was resuspended in 0.2 mL of SDS sample solubilization buffer. The sample was sonicated with an ultrasonic homogenizer (three 10-s bursts at 100 W and 4 °C).

The disrupted cells were centrifuged in an Eppendorf microfuge at maximum speed (14,000 *g*) for 30 minutes at 4°C. For SDS-PAGE experiments the supernatant was collected and resuspended in an equal volume of buffer containing 0.5 M Tris HCl pH 8.0, glycerol, SDS and bromophenol blue.

V.2.3.4. SDS-PAGE and staining

SDS-PAGE was performed on vertical gels (12.5%T and 0.97%C) in a Hoefer™ SE 600 Ruby® (Amersham Biosciences) unit, according to the procedure described by Laemmli (1970) (Laemmli, 1970) with some modifications. Electrophoresis was carried out at constant amperage of 30 mA per gel until the dye-front reached the bottom of the gels that were then stained with Coomassie Brilliant Blue R250 and washed in water overnight. Gels were then fixed in 6% trichloroacetic acid for 4 h and in 5% glycerol for 2 h (Igrejas, 2000).

V.2.3.5. Two-dimensional electrophoresis and proteomics analysis

Immobiline™ pH Gradient (IPG) technology (Görg et al., 2007) was used for 2-DE (O'Farrell, 1975). Protein samples from the wild-type strain were compared to protein samples from the stressed strain by subculture at the minimal inhibitory concentration of CTX. For isoelectric focusing (IEF), precast 13-cm IPG strips with linear gradient of pH 3-10 (pH 3–10 NL, Amersham Biosciences, UK) were passively rehydrated overnight in a reswelling tray with rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte IPG buffer pH 3-10) at room temperature. IPG strips were covered with DryStrip Cover Fluid (Plus One, Amersham Biosciences). Lysis buffer (9.5 M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3-10) and 10 mM Pefabloc® proteinase inhibitor) was added to the bacteria (1:1, v/v). Samples containing a total of 100 µg of protein were cup loaded on to the rehydrated IPG strips (Görg et al., 2000) and proteins were focused sequentially at 500 V for 1 h, 1000 V for 8 h, 8000 V for 3 h and finally 8000 V for 1 h 30 min incremented to 23135 V/h on an Ettan™ IPGPhor II™ (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed according to Görg et al. (2007) and the GE Healthcare protocol for 13-cm IPG strips pH 3-10 to optimize the running conditions, resulting in a final 13 h 30 min run. Focused IPG strips were then stored in plastic bags at -80ºC. Before running the second dimension, strips were incubated twice 15 min in equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer pH 8.8). For the first equilibration 1% DTT and bromophenol blue were added to the equilibration buffer and for the second 4% iodoacetamide and bromophenol blue were added. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied to 12.52% polyacrylamide gels in a Hoefer™ SE 600 Ruby® (Amersham Biosciences) unit. IPG strips were inserted in the stacking gel for SDS-PAGE using a method modified to improve protein resolution (Igrejas, 2000; Laemmli, 1970). Gels were fixed in 40% methanol/10% acetic acid for 1 h then

stained overnight in Coomassie Brilliant Blue G-250 (Görg et al., 2004). Coomassie-stained gels were scanned on a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA) and the resulting digitized images were analyzed using Image Master 5.0 software (Amersham Biosciences; GE Healthcare).

V.2.3.6. Protein digestion

The excess of staining was removed from the gel, and the proteins were located. The spots from 2D-PAGE were excised manually with sterile tips under sterile dust-free environment and transferred to individual tubes for enzymatic digestion. For the protein digestion, the gel pieces were incubated with trypsin at 37ºC overnight. Concerning the peptide extraction, the reaction media was acidified with Formic acid (FA) to stop the enzymatic digestion. Then, the supernatant containing the digested peptides was removed to another tube, and additional peptides were extracted from the gel with a 50 % acetonitrile/0.1 % TFA solution. After the extraction step, the supernatant and the extraction solutions were combined and analyzed by MS, or stored frozen for further analysis.

V.2.3.7. Matrix formulation and sample deposition

Matrix solution was prepared by dissolving 10 mg of α-CHCA in 1mL of H_2O/acetonitrile/TFA (49.95:49.95:0.1). The sample (10 µL) was mixed with 10 µL of the α–CHCA matrix solution with vortexing for 30 s. One microliter of the mixture was spotted on a MALDI-TOF MS target plate and allowed to dry for 5 min.

V.2.3.8. MALDI-TOF MS analysis

An Ultraflex II MALDI-TOF/TOF-MS instrument from Bruker Daltonics equipped with a 200 Hz Smart beam laser system, was used to obtain peptide mass fingerprints (PMF). MALDI mass spectra were acquired as recommended by the manufacturer. Measurements were taken in reflector positive ion mode in the mass range of 600-3500 Da. Close external calibration was performed with the monoisotopic peaks of the bradykinin (757.3992), angiotensin II (1046.5418), angiotensin I (1296.6848), substance P (1347.7345), bombesin (1619.8223), renin substrate (1758.9326), ACTH clip 1-17 (2093.0862), ACTH 18-39 (2465.1983), and somatostatin 28 (3147.4710). Mass spectrum analysis for each sample was based on the average of 500 laser shots. The peak lists were generated from the mass spectra using the peak detection algorithm SNAP from the FlexAnalysis 3.3 software (Bruker Daltonics). Peptide mass fingerprints were searched with the MASCOT search engines. MASCOT parameters were as follows: variable modifications, oxidation (M); fixed modifications, carbamidomethyl (C); Swiss-Prot database; one missed cleavage allowance, and a peptide tolerance of up to 100 ppm. The default significance threshold, $p < 0.05$, was used. A match was considered

successful if the protein identification score was located outside the random region and the protein analysed scored in the first position. Figure V.2.1 summarises the methodology used in the present study.

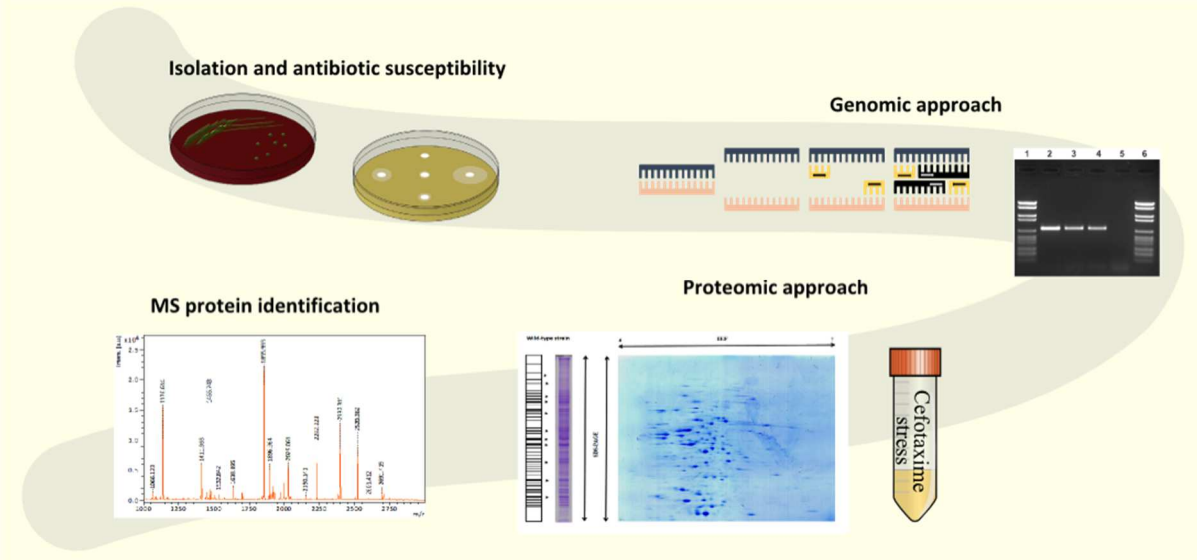

Figure V.2.1 | Typical genomic and proteomic workflow representing the classical components of genetical study and protein identification

V.2.4. Results

In this study a comparative proteomic analysis was performed between strain C5478 without antimicrobial stress (wild-type) and C5478 stressed with cefotaxime.

A stress or shock involves a change from one environmental condition to another, so the natures of both the original condition and the shock are important in defining the appropriate response. Here, we reasoned that comparing the proteomes of a naturally occurring ESBL-producing *E. coli* strain (C5478 wild type strain) and the ESBL-producing *E. coli* strain stressed by CTX (stressed strain) would reveal key differences that might be necessary for bacterial colonization in the presence of β-lactams. We used 2D-PAGE and MALDI-TOF MS to initially screen for significant differences in the distribution of proteins between wild-type and stressed strains (Tables S9 and S10).

The comparative proteomic analysis revealed numerous protein species abundance changes in the stressed strain by CTX in relation to the wild-type strain. A total of 188 spots were excised from the 2-DE gel of the wild-type strain and 112 were successfully identified by MS, representing 110 different proteins (Table S9). Concerning the 2-DE gel of the stressed strain, 171 spots were excised and 156 were identified, representing 143 different proteins (Table S10). The proteins identified in the 2 strains were categorized with reference to their biological functions. These

proteins were involved in metabolism, protein synthesis, cell division, stress response, antimicrobial resistance, transport, unknown functions, among others (Figure V.2.2).

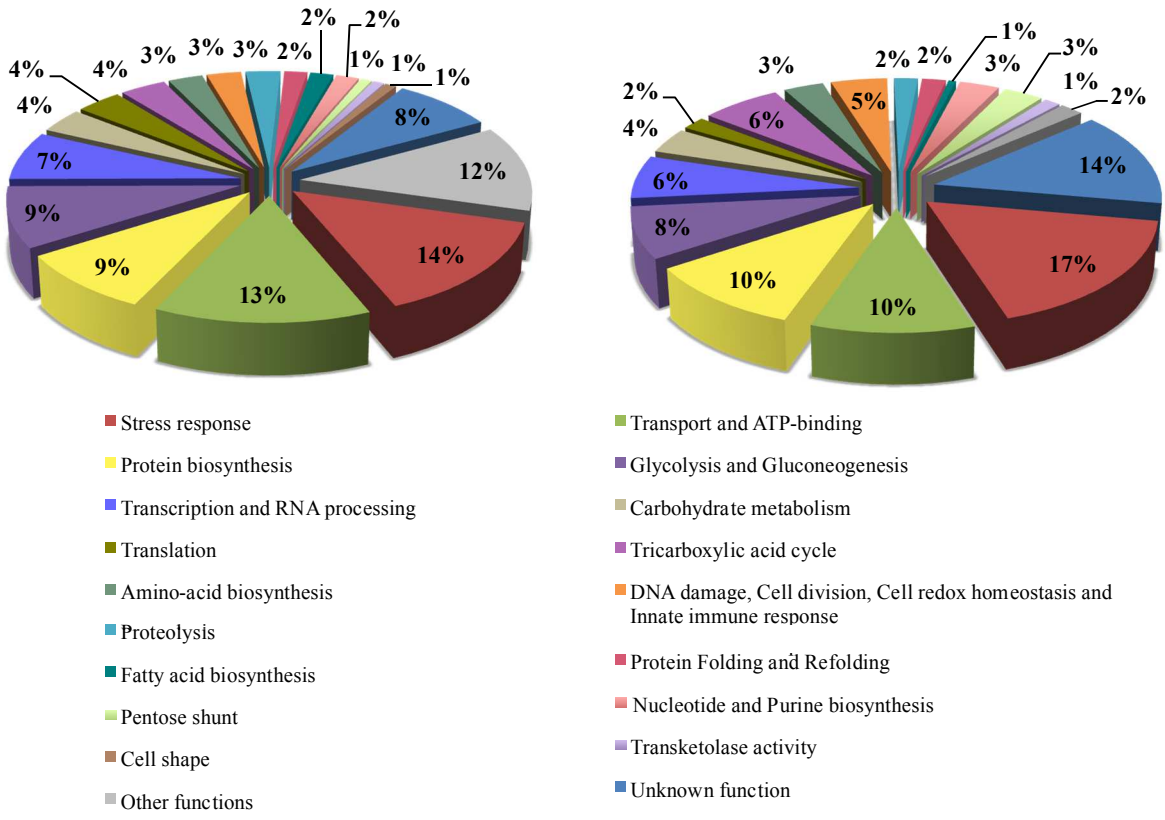

Figure V.2.2 | Distribution of biological functions of the two strain proteins
Left wild type strain and right stressed strain

Various proteins were found in multiple spots on the 2 gels. From the gel of wild-type strain, Mdh (spots 74 and 117), Tuf1 (spots 110, 127 and 128), YajQ (spots 36, 56 and 157), GpmA (spots 46 and 50), PflB (spots 169 and 172), YeaD (spots 76 and 80), GadB and GadA (spots 114 and 126), DaaA (spot 32 and 34); and in the gel of stressed strain, Tsf (spots 243, 337), Dps (spots 198, 200), Pgk (spots 268, 339 and 344), Pnp (spots 303 and 316), rpoB (spots 252 and 315), Gnd (spots 280 and 345), PflB (spots 327 and 328), SlyA (spots 213 and 262), Prs (spots 285 and 360), Tpx (spots 205 and 281), TalA (spots 253 and 254), CysK (spots 253 and 254), MalE (spots 243, 266 and 338), Ssb (spots 207 and 208), AcnA (spots 322 and 323), TktA (329 and 330) and ClpB (spots 312 and 331), migrated with different charges and masses during electrophoresis. A distinctive position in a 2-DE gel had to be the result of a different chemical structure of the protein. Each covalent chemical alteration of a protein results to a new protein species. Hence, these spots could correspond to different protein species with posttranslational alterations (dos Santos et al., 2010; Jungblut et al., 2008).

A total of 268 proteins were distinctively expressed in both samples. The proteins that decreased in percentage under CTX stress (Figure V.2.3) were principally proteins involved in transport and ATP-binding (like ArgT, FliY and NmpC), glycolysis and gluconeogenesis (like GapA, GpmA, PykF and OckA), transcription and RNA processing (like DaaA, RopB, Fur and Pnp), translation (like RpsA and RpsB), proteolysis (PepQ and PrlC), fatty acid biosynthesis (like AccC and FabF), and cell shape (DdlB).

In contrast, the percentage of stress response proteins expressed was much greater in bacteria under CTX stress (Figure V.2.4) such as those involved in mechanisms of acid resistance (GadA, GadB, WrbA), oxidative stress (like Tpx, SodB and OsmC), other general cellular stress responses involving cold and heat shock proteins (DnaK, ClpB, ClpP). Among the numerous changes noted in the stressed strain, the percentage of proteins related to DNA damage, cell division, cell redox, innate immune response (like FtsZ, FtsY, SsB and YbbN), starvation (Dps) and antimicrobial resistance (OmpX, FabI) increased. In the stressed bacteria, there were also increased percentages of enzymes involved in protein biosynthesis (like AspS, FusA and GlyS), the tricarboxylic acid cycle (like MdH, SucB and SucC), amino-acid biosynthesis (like CysK and CarB), nucleotide and purine biosynthesis (PurA and Prs), the pentose shunt (like TalA, TalB and GnD) and transketolase activity (TktA) among others.

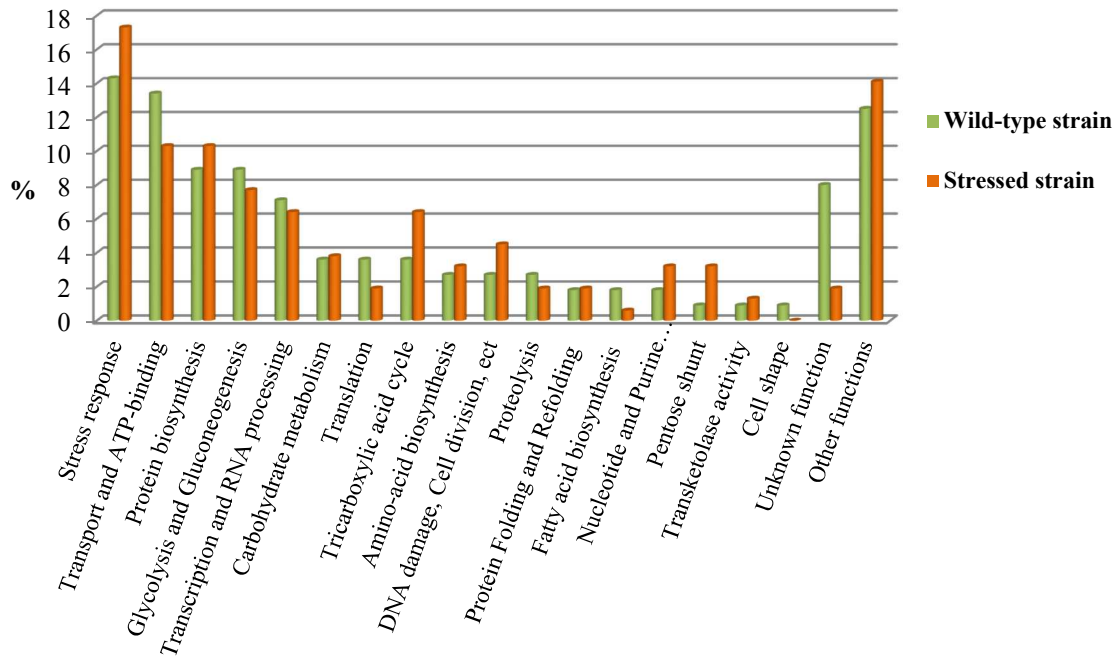

Figure V.2.3 | Percentage distribution by biological function of proteins identified in the wild type and CTX stressed *E.coli* C5478 strains

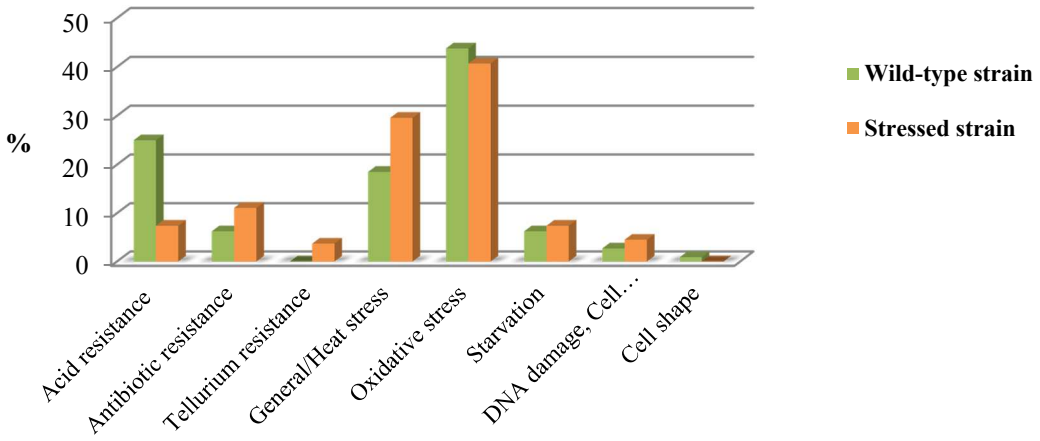

Figure V.2.4 | Percentage distribution of stress response proteins identified in wild type and CTX stressed *E. coli* C5478 strains

V.2.5. Discussion

The widespread use of antimicrobials to treat infections means that cases of bacterial resistance to these drugs are becoming more frequent, presenting a continuing challenge in medicine. Multidrug resistance is a main cause of clinical failure in treating bacterial infections. Increasing evidence suggests that bacteria can resist multiple antimicrobials through intrinsic mechanisms that rely on gene products such as efflux pumps that expel antimicrobials and specific membrane proteins that block the penetration of drug molecules.

The response of bacterial metabolism to environmental perturbations is characterized by a fast and appropriate adjusting of physiology on every level of the cellular and molecular network. The entire sequence of the *E. coli* genome has been available since 1997 greatly facilitating proteomic methodologies of 2-DE with MALDI-TOF MS analysis of proteins related to antimicrobial resistance of this bacterium.

In CTX-stressed bacteria, there were mainly decreases in percentages of proteins involved in transport/ATP-binding, glycolysis, transcription/RNA processing, translation, proteolysis, fatty acid biosynthesis and also some proteins related to oxidative and acid stress responses. Though enzyme analyses and other physiological data are still required, we can make some tentative conclusions about these overall changes in bacterial physiology. The consequences of decreased protection against oxidative stress and acid stress may have been compensated for by increased levels of proteins active in DNA damage repair, cell division, cell redox homeostasis, the innate immune response as well as proteins associated with antimicrobial resistance, tellurium resistance, general/heat stress and starvation. The stressed bacteria had acquired defence mechanisms normally related to the stress response, and an increased tolerance to antimicrobials was confirmed. Increases in the percentages of proteins involved in the stress response are often at the expense of proteins

implicated in translation. Similarly we found that the increase in tolerance to cefotaxime in the stressed strain has deleterious effects on ribosomal activity. These metabolic modifications could indicate the biological cost of antimicrobial resistance, though it is possible that other changes in these proteins that were not determined on these 2-DE gels could explain the results. Any such costs are likely to be mitigated by subsequent evolution as described by Andersson (2003).

V.2.5.1. Stress response

Stress responses are usually a combination of specific responses aimed at minimizing deleterious effects (e.g. catalase production during oxidative stress) or repairing damage (e.g. chaperone expression under temperature stress) and general responses.

The results of this study revealed a significant increase in the percentage of stress response proteins, like GadA and GadB, present in the naturally occurring and stressed bacteria. GadA and GadB are the most efficient acid resistance system (Castanie-Cornet et al., 1999). Defences against oxidative stress involve constitutive and tightly regulated adaptive mechanisms to prevent and scavenge oxidants as well as to repair damaged biomolecules. The widely accepted simplified paradigm of defence against oxidative stress is that superoxide is removed by superoxide dismutases such as SodB (spots 26 and 209) generating hydrogen peroxide, which is removed by catalases, like KatE (spots 174 and 324) and peroxidases like AhpC (spot 212, MW 20862 and PI 5.03). Several of these defences are controlled by regulators that respond to iron such as Fur (spot 2, MW 17012 and PI 5.68) or oxygen tension such as ArcA (spot 52) (Seib et al., 2006).

In the CTX-stressed bacteria, besides the ribosome-associated Trigger factor Tig (spot 284), the cytoplasm of the strain contains two of the three FK506-binding proteins (FKBPs) SlyD (spot 240) and FklB (spot 215). FKBPs are enzymes grouped into a superfamily of peptidyl-prolyl-cis/trans-isomerases (PPIases) that act as chaperones in folding proteins. Many proteins involved in disease processes require modification, thus PPIases can play an important role in pathogenesis (Seib et al., 2006).

Other proteins related to oxidative stress defence in the stressed strain were identified here such as CysQ (spot 219), YdhR (spot 323), YhfA (spot 191), MaeB (spot 310) and Tpx (spots 205 and 281). The osmotically inducible protein OsmC, which was identified in both strains (spots 7 and 190), is involved in hydroperoxide protection particularly in host–pathogen interactions (Mongkolsuk & Helmann, 2002).

Some heat shock proteins (HSPs) function as molecular chaperones or have functions linked with DNA replication, cell division, and maintenance of active protein conformations (Mason et al., 1999). The number of proteins related to heat stress increased under CTX stress, for example ClpB (spots 312 and 331) and HtpG (spot 293), which participate in protein folding in stressed *E. coli* cells (Thomas & Baneyx, 2000).

KlaB (TelA, MW 42130 and PI 6.05) was one of the stress response proteins identified in the stressed strain (spot 287). KlaB is responsible for tellurium resistance (Tables S9 and S10). Tellurite is toxic to *E. coli* through an unknown mechanism that involves an increase in oxidative stress. It may replace sulfur in various proteins, rendering them non-functional.

V.2.5.2. DNA damage, cell division, redox homeostasis and immune response

In the stressed strain derived from C5478, there were increased percentages of proteins related to DNA damage, cell division and cell redox homeostasis. Of the cell division proteins, FtsZ found in the stressed strain (spot 261) is of great interest as it is the earliest actor in the division pathway and its level dictates the frequency of division (Bi & Lutkenhaus, 1991). Furthermore, FtsY, also identified in the stressed strain (spot 314, MW 54480 and PI 4.46), is a signal recognition particle receptor in *E. coli* that mediates the targeting of integral membrane proteins to translocons by interacting with both signal recognition particle (SRP)-nascent polypeptide–ribosome complexes and the cytoplasmic membrane (Maeda et al., 2008).

The SsB protein, one of the proteins in the DNA damage category, was found in the stressed strain in two spots (207 and 208). SsB protects and sequesters single-stranded DNA regions until the double helix can be reformed (Bore et al., 2007).

The relative amounts of cell wall proteins found in the stressed bacteria are quite high. Constant turnover of the cell wall provides ample decoy material for *E. coli* to engage host innate defences and the rate of cell wall production is directly proportional to the rate of colonization of the human tract (Muthukrishnan et al., 2011). Penicillin-binding proteins (PBPs), the target enzymes of β-lactam antimicrobials such as cefotaxime, play an essential role in bacterial cell elongation, cell division and cell wall biosynthesis. PBPs are membrane-bound enzymes that are widespread in *E. coli* (Zhao et al., 1997).

The SOS response supports bacterial propagation by inhibiting cell division during repair of DNA damage and cell survival. The extent of induction of the SOS response can be determined by measuring β-galactosidase synthesis from a LacZ (spot 319, MW 117300 and PI 5.20) fusion with the SOS-regulated promoter of the *sfi*A gene, which prevents FtsZ (spot 261, MW 40299 and PI 4.63) polymerization and inhibits cell division when SOS is activated (Maeda et al., 2008). The immediate response of bacterial pathogens to antimicrobial therapy is of key interest when considering how to deal with infectious diseases.

The FtsZ protein, identified in the stressed bacteria, is crucial to the cell-division process. It assembles as a dynamic ring on the inner surface of the cytoplasmic membrane at the place where division will happen, with the formation of the ring being the signal for septation to begin (Bi & Lutkenhaus, 1991).

It is important to also point out the presence of the FtsY protein in the stressed bacteria. The capability of transformants producing chimeric FtsY to process β-lactamase was evaluated in a

recent study (Maeda et al., 2008). While β-lactamase is not dependent on signal recognition particle (SRP) for export, it does require SRP function for cleavage of the signal peptide, as leader peptidase is an SRP-dependent membrane protein in *E. coli* (Beha et al., 2003).

V.2.5.3. Antimicrobial resistance

More of the antimicrobial resistant proteins Ompx and FabI were observed in the stressed strain. OmpX is present in the both strains (spot 14, MW 67648 and PI 5.30) and (spot 196, MW 18648 and PI 5.30). Because of the function of this integral outer membrane protein in membrane permeability, OmpX may be involved in controlling the penetration of antimicrobials such as β-lactams and fluoroquinolones through the enterobacterial outer membrane as is known for chloramphenicol, tetracycline, and kanamycin (Dupont et al., 2007). OmpX belongs to a family of highly conserved bacterial proteins that have been assigned key functions in promoting bacterial adhesion and entry into mammalian cells (Mecsas et al., 1995).

Usually, resistance to fluoroquinolones arises spontaneously because of point mutations that result in amino acid substitutions within the topoisomerase subunits GyrA, GyrB, ParC or ParE, a decreased expression of outer membrane porins, or an overexpression of multidrug efflux pumps (Hammerum & Heuer, 2009). GyrB was identified (spot 325, MW 90179 and PI 5.72) in this study, which is consistent with the quinolone acid resistance observed in the strain. The wild-type strain was known to have two amino acid changes in GyrA (S83L+D87G) and one in ParC (S80I) responsible for ciprofloxacin and nalidixic acid resistance (Radhouani et al., 2012a).

The presence of the protein FabI (enoyl reductase), an important enzyme in fatty acid biosynthesis, was observed in the stressed bacteria (spot 245, MW 28074 and PI 5.58). Triclosan, which has been described as a broad-spectrum antimicrobial and is found in many antibacterial consumer products, is now known to specifically target FabI in *E. coli* (Knobler et al., 2003).

The likely loss of energy production in the stressed strain caused by the decreased percentage of proteins involved in the glycolytic pathway may be a cost that resistance imposes on the fitness of bacteria (dos Santos et al., 2010). Besides glycolysis, other pathways such as the tricarboxylic acid (TCA) cycle are likely to be involved in regulating and responding to CTX bactericidal effects, which may explain the increase in the amount of TCA cycle related proteins. Further study of the relationships between iron, metabolism, and iron-sulfur cluster-containing proteins (Hantke, 2001) is necessary to identify any posttranscriptional events not captured in gene expression studies that trigger this common cell death pathway (Kohanski et al., 2007).

V.2.5.4. Transport proteins

The wild-type strain is multidrug resistant. Both the wild-type strain and the stressed strain can resist antimicrobial agents by active efflux of the agents using translocation machinery (Hassan

et al., 2007). This is borne out by our results showing that some of the most abundant proteins, more than 13% and 10% of those identified in the non-stressed and stressed conditions respectively, were linked to transport and ATP-binding functions (Figure V.2.3). These included three proteins related to ATP-binding detected in the stressed strain, Acs (spot 329), GlnA (spot 295) and PpsA (spot 304). The ATP synthase subunit alpha (AtpA) and beta (AtpD) were present in the wild-type and stressed strain. In *E. coli* ATP synthase activity provides proton motive force for efflux pump activity (Coldham & Woodward, 2004). Additionally, the SecD protein identified in the stressed strain (spot 202, MW 66648 and PI 8.62) is a transport protein implicated in bacterial pathogenesis and in the secretion of virulence proteins (Muthukrishnan et al., 2011).

Antimicrobial resistance often entails a metabolic cost because the resistance mutations typically occur in genes of target molecules that have essential functions in the cell. In fact, mutation is a primary cause of bacterial resistance to antimicrobials. In *E. coli* active efflux systems are common mechanisms of reduced susceptibility to fluoroquinolones that may confer resistance, particularly when associated with mutation in GyrA, GyrB or ParC (Coldham & Woodward, 2004) like those in GyrA and ParC in the wild-type C5478 strain studied here.

As previously mentioned the enzyme FabI was identified in the stressed bacteria. Given the multi-drug efflux pumps in *E. coli*, overexpression of one of the pumps could participate in resistance mediated by a FabI mutation, which might have the effect of spreading antimicrobials.

V.2.5.5. Amino acids and protein biosynthesis/metabolism

The number of proteins related to amino acids, protein biosynthesis and metabolism increased in the stressed bacteria. One example is the CysK protein (spots 253 and 254) that has elsewhere been shown to mediate tellurite resistance in *E. coli*. Pyridoxine 5'-phosphate synthase (PdxJ, spot 235) was present in the stressed bacteria. The genes *pdx*J and *pdx*A encode proteins involved in vitamin B_6 biosynthesis. It was proposed that PdxJ enzymes could be ideal therapeutic targets against bacterial pathogens (Grubman et al., 2010).

Transaldolase A (TalA), found in the stressed bacteria (spot 253 and 254), is involved in the nonoxidative branch of the pentose phosphate pathway. The gene coding for the isozyme TktA is also detected in the stressed strain (spots 229 and 230) and is known to be upregulated by stress conditions (Domain et al., 2007).

The Upp protein was found in the stressed bacteria (spot 214). It was shown that energy production genes such as SucB are involved in persister survival and tolerance of multiple antimicrobials and stresses in *E. coli* (Ma et al., 2010). Upp, expressed naturally in *E. coli*, converts the chemotherapeutic drug 5-fluorouracil (5-FU) into 5-fluorouridine monophosphate, an extremely toxic compound. Adenoviral delivery of the Upp gene to human cancer cell lines increased their sensitivity to 5-FU (Kanai et al., 1998). The *E. coli* enzymes DeoD (spots 33, 217) have also been used in gene therapy studies (Kanai et al., 1998).

The main HSPs DnaK, DnaJ, GroEL are molecular chaperones that assist in the correct folding and assembly of proteins and are implicated in various cellular processes including DNA replication, RNA transcription, flagella synthesis and UV mutagenesis (Yura et al., 1993). HSPs participate in the immune response to bacterial infections and the development of autoimmune diseases (Murray & Young, 1992). Different classes of HSPs from different bacteria can directly induce cytokine expression and secretion in macrophages (Retzlaff et al., 1994). We found several chaperones related to the stress response were found in the both strains but they were more abundant in the stressed strain. It has been demonstrated previously that β-lactamase interacts with GroEL and GroES (Bochkareva et al., 1988; Laminet et al., 1990) and that export of β-lactamase is defective in GroEL and GroES mutants (Beha et al., 2003).

Glycopeptides such as vancomycin and teicoplanin target the peptidoglycan cell wall of bacteria by selectively binding to the D-alanyl–D-alanine termini of peptidoglycan precursors preventing cross linking to adjacent strands. The presence of DdlB, an attractive target for developing novel antimicrobials (Triola et al., 2009), was detected in the wild-type strain.

V.2.6. Conclusion

In the present study the use of 2D-MALDI-TOF MS provided wide proteome coverage as demonstrated by the number of proteins identified (112 in the wild-type strain and 156 in the stressed strain). This made it possible to more comprehensively evaluate changes in protein expression in response to selection with CTX. The numerous changes in protein levels observed in the stressed strain suggest that resistant micro-organisms may adapt to adverse environmental conditions (Justice et al., 2008; Linares-Rodriguez & Martinez-Menendez, 2005) with implications for several aspects of bacterial metabolism, which may be mirrored in their virulence parameters.

The ability of resistant bacteria to survive in a population and in the community depends on numerous factors, such as biological fitness. In this case, antimicrobial resistance can be stabilized with fitness-restoring compensatory mutations and may permit completely resistant strains to compete successfully with susceptible strains in an antimicrobial-free environment (Andersson, 2003). In conclusion, we have demonstrated that a wide range of proteins change in abundance in a diverse secondary response to treatment with cefotaxime, a third-generation cephalosporin. For the proteins already associated with antimicrobial resistance, such as OmpX, FtsZ and FtsY, the modifications are likely due to the immediate effects of encountering CTX. These findings will be helpful for understanding more generally what constitutes a functional proteome and, in particular, how antimicrobial-resistant mechanisms work.

V.2.7. Supplementary material

Supplementary material regarding this manuscript is in attachments (Tables S9 and S10).

V.2.8. Acknowledgements

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V.2.9. References

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CHAPTER VI

PROTEOMIC CHARACTERIZATION OF ANTIMICROBIAL-RESISTANT ENTEROCOCCI ISOLATES IN WILD ANIMALS

CHAPTER VI

VI.1. Radhouani H, Poeta P, Pinto L, et al. (2010) **Proteomic characterization of *van*A-containing *Enterococcus* recovered from Seagulls at the Berlengas natural reserve, W Portugal.**

VI.2. Radhouani H, Poeta P, Pinto L, et al. (2012) **Comparative proteomic map among *van*A-containing *Enterococcus* isolated from yellow-legged gulls.**

VI.1. Proteomic characterization of *van*A-containing *Enterococcus* recovered from Seagulls at the Berlengas Natural Reserve, W Portugal

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VI.1.1. Abstract

Background: Enterococci have emerged as the third most common cause of nosocomial infections, requiring bactericidal antimicrobial therapy. Although vancomycin resistance is a major problem in clinics and has emerged in an important extend in farm animals, few studies have examined it in wild animals. To determine the prevalence of *van*A-containing *Enterococcus* strains among faecal samples of Seagulls (*Larus cachinnans*) of Berlengas Natural Reserve of Portugal, we developed a proteomic approach integrated with genomic data. The purpose was to detect the maximum number of proteins that vary in different enterococci species which are thought to be connected in some, as yet unknown, way to antibiotic resistance.

Results: From the 57 seagull samples, 54 faecal samples showed the presence of *Enterococcus* isolates (94.7%). For the enterococci, *E. faecium* was the most prevalent species in seagulls (50%), followed by *E. faecalis* and *E. durans* (10.4%), and *E. hirae* (6.3%). *Van*A-*containing* enterococcal strains were detected in 10.5% of the 57 seagull faecal samples studied. Four of the *van*A-containing enterococci were identified as *E. faecium* and two as *E. durans*. The *tet*(M) gene was found in all five tetracycline-resistant *van*A strains. The *erm*(B) gene was demonstrated in all six erythromycin-resistant *van*A strains. The *hyl* virulence gene was detected in all four *van*A-containing *E. faecium* isolates in this study, and two of them harboured the *pur*K1 allele. In addition these strains also showed ampicillin and ciprofoxacin resistance. The whole-cell proteomic profile of *van*A-containing *Enterococcus* strains was applied to evaluate the discriminatory power of this technique for their identification. The major differences among species-specific profiles were found in the positions corresponding to 97-45 kDa. Sixty individualized proteins for each *van*A isolate was identified and suitable for peptide mass fingerprinting measures by spectrometry measuring (MALDI/TOF MS) and their identification through bioinformatic databases query. The proteins were classified in different groups according to their biological function: protein biosynthesis, ATP synthesis, glycolysis, conjugation and antibiotic resistance. Taking into account the origin of these strains and its relation to infectious processes in humans and animals, it is important to explore the proteome of new strains which might serve as protein biomarkers for biological activity.

Conclusions: The comprehensive description of proteins isolated from vancomycin-resistant *Enterococcus faecium* and *E. durans* may provide new targets for development of antimicrobial agents. This knowledge may help to identify new biomarkers of antibiotic resistance and virulence factors.

VI.1.2. Introduction

Enterococcus spp. are commensal bacteria of the intestinal microbiota of humans and animals but are now becoming recognized as important causes of nosocomial, and to a lesser extent, community acquired infections. Typical enterococcal infections occur in hospitalized patients with underlying conditions representing a wide spectrum of severity of illness and immune modulation (Franz et al., 2001; Pillar & Gilmore, 2004). The emergence of vancomycin-resistant enterococci (VRE) in Europe has been associated with the use of avoparcin as feed additive in food animals (Bager et al., 1997), until its ban in 1997 by the European Union. There are reports on the presence of VRE in farm animals in different countries (Bager et al., 1997; Devriese et al., 1996; Novais et al., 2002; Torres et al., 2003), including in Portugal, but studies dealing with the occurrence of VRE in wild animals are limited (Poeta et al., 2007; Poeta et al., 2005a). For many years, vancomycin was considered as the last resort when all other classes of antibiotics failed. In the nineteen-eighties plasmid-mediated resistance against vancomycin among enterococci was first demonstrated and since then occurrences of infection caused by VRE have increased dramatically. This situation causes several challenges, including firstly the sole availability of expensive new antimicrobials for therapy of VRE infections since most strains are also resistance to multiple other economically acceptable drugs in developing countries, e.g., aminoglycosides or ampicillin, and secondly the possibility that the vancomycin resistance genes present in VRE could be transferred to other Gram-positive microorganisms such as *Staphylococcus aureus* (CDC, 1995). On the other hand virulence factors have been mainly detected in bacteria of the *E. faecalis* species, being *E. faecium* generally free of these determinants (Eaton & Gasson, 2001). Studies reporting the presence of virulence factors in enterococci of food and animal origin are few (Eaton & Gasson, 2001; Mannu et al., 2003), and the occurrence is not well documented in faecal enterococci from wild animals (CDC, 1995; Poeta et al., 2005b; Poeta et al., 2008). Birds are sentinel species whose plight serves as barometer of ecosystem health and alert system for detecting global environmental ills. Harmful effects seen in wildlife can be useful 'sentinel events' warning us of potential hazards for humans. This calls for integrated ecological and health hazard appraisals. Frequently, these wild birds are often opportunistic marine feeders along the shoreline or offshore, but also readily utilizing the food sources provided by humans, especially garbage. Migrating birds that fly and travel long distance seem to act as transporters, or as reservoirs, of resistant bacteria and may consequently have a significant epidemiological role in the dissemination of resistance, as well as being mirrors of the spectrum of pathogenic microorganisms present in humans. Of such migratory birds, particularly dominant in our study were the seagulls.

A natural heritage of great environmental value, the Berlengas archipelago, is situated about 10 km from the Portugal Peniche coast. It comprises the Berlengas Grande Island and adjacent reefs. It has been classified as a Natural Reserve since 1981. In an almost pure and wild state, the

archipelago is a rich habitat for many animals and plant species. The dominant local fauna consists mainly of sea birds. Nowadays, the archipelago has no permanent human population and is only visited by scientists and, in the summer, by a small number of tourists. Visitors are required to respect the natural environment and the species that inhabit the area. Makeshift paths are marked with stones and park rangers watch out for visitors straying into the prohibited areas, disturbing the birds and the wildlife of this reserve. For these reasons, apparently, the seagulls are not directly under antibiotic selective pressure in the Berlengas.

Polyacrylamide gel electrophoresis (PAGE) of whole-cell polypeptides solubilised by treatment with sodium dodecyl sulfate (SDS) has been used to identify and type bacteria (Coleri et al., 2004; Hook et al., 1991; Jackman, 1985). This technique allows the comparative study of large numbers of proteins encoded by a significant portion of the genome and, therefore, has a very high potential for measuring relationships among isolates (Elliott et al., 1990; Hook et al., 1991; Wang et al., 2010). Over the past decade numerous genomes of pathogenic bacteria were fully sequenced and annotated, while others are continuously being sequenced and published. More recently to understand the molecular mechanisms of bacteria resistance to glycopeptides, proteomic profiles of vancomycin-resistant *Enterococcus faecalis* V583 (reference strain) and V309 (clinical isolate) were analysed (Wang et al., 2010). Vancomycin induced specifically and reversibly VanA, VanX, VanB, and VanXB. Some of these proteins have known vancomycin resistance functions or are related to virulent factors, stress, metabolism, translation, and conjunction, which would help *Enterococcus* survive under drug selection.

The genetic characterization of antimicrobial resistance genes as well as their location and diversity is important in identifying factors involved in resistance, understanding the diversity of multidrug resistant strains, identifying genetic linkages among markers, understanding potential transfer mechanisms, and developing efficient detection methods. The aim of this study was to analyse the prevalence of faecal carriage by *van*A-containing *Enterococcus* strains in seagulls (*Larus cachinnans*) inhabiting the Berlengas archipelago, which are a group of very small islands of the Portuguese coast near to the city of Peniche. The islands are one of the first protected areas in the world. Additionally *esp* and *hyl* virulence factor genes were also investigated. Unlike genome studies, investigations at the proteomic level provide insights into protein abundance and/or post-translational modifications and it is also one of the best methods of investigating basic biological processes such as pathogenesis, physiology, and metabolic mechanisms. For these reasons the whole-cell protein (WCP) profiles of *van*A-containing *Enterococcus* strains was followed by genotypic and proteome characterization of these bacteria. The goal was to demonstrate the usefulness of the WCP profiling approach as a technique for identifying, typing and studying the relationships between isolates.

VI.1.3. Material and methods

VI.1.3.1. Samples and bacteria

The presence of faecal VRE was investigated in 57 faecal samples recovered from seagulls of Berlengas islands. Faecal samples of seagulls were recovered in the soil along the entire Berlengas Island during September of 2007 and they were tested for the presence of *van*A-containing *Enterococcus* isolates.

Faecal samples were diluted and sampled in Slanetz-Bartley agar plates, incubated 48 h at 35°C, and two different colonies were isolated but only a single isolate of each species was included. Colonies with typical enterococcal morphology were identified to the genus and species level by cultural characteristics, Gram's strain, catalase test, bile-aesculin reaction and by biochemical tests using the API ID20 Strep system (BioMérieux). Species identification was confirmed by PCR using primers and conditions for the different enterococcal species (Torres et al., 2003).

VI.1.3.2. Antimicrobial susceptibility testing

Antibiotic susceptibility was tested for 11 antibiotics of interest in animal and human medicine (μg/disk): vancomycin (30), teicoplanin (30), ampicillin (10), streptomycin (300), gentamicin (120), kanamycin (120), chloramphenicol (30), tetracycline (30), erythromycin (15), quinupristin-dalfopristin (15), and ciprofloxacin, (5), by the disk diffusion method (CLSI, 2007). Antibiotic disks were obtained from Oxoid (Oxoid Ltd, Basingstoke, UK), with the exception of aminoglycoside disks that were prepared in the laboratory. Minimal inhibitory concentrations (MICs) of vancomycin (Eli Lilly, Indianapolis, IN, USA) and teicoplanin (Hoeschst Marion Roussell, Paris, France) were determined by the agar dilution method according to the CLSI (CLSI 2007). Serial two-fold dilutions were tested for antibiotic MIC determinations (from 0.25 μg/mL to 64 μg/mL). The breakpoints for resistance were the following ones: vancomycin or teicoplanin, ≥32 μg/mL (a MIC of 8-16 μg/mL of vancomycin or 16 μg/mL of teicoplanin was considered as intermediate susceptibility). Only high-level resistance to aminoglycosides was considered in the susceptibility of our enterococci. *E. faecalis* strain ATCC29212 and *Staphylococcus aureus* strain ATCC29213 were used for quality control.

VI.1.3.3. Antimicrobial resistance genes

Vancomycin resistance genes (*van*A, *van*B, *van*C-1, *van*C-2/3 and *van*D) were tested by PCR in all vancomycin-resistant enterococcal strains, and positive amplicons were sequenced (Torres et al., 2003). Resistance genes for other antibiotics, including *tet*(M), *tet*(L), *erm*(A), *erm*(B), *erm*(C), were analysed by PCR (Torres et al., 2003). The presence of *esp* and *hyl* virulence factor genes was tested by PCR in all isolates, using primers and conditions previously described (Klare et al., 2005), and the *purK* allele type was investigated by PCR and sequencing in all *E. faecium*

isolates. Positive and negative controls were included in all analyses and the bacteria come from the collection of the University of Rioja (Spain). The specific gene harboured by the positive controls used in this study had previously been confirmed by sequencing, in all the cases.

VI.1.3.4. MLST typing

*E. faecium van*A isolates were characterized by Multilocus Sequence Typing (MLST). For this purpose, internal 400- to 600-bp fragments of seven housekeeping genes (*adk*, *atp*A, *ddl*, *gdh*, *gyd*, *pur*K and *pst*S) were amplified and sequenced. The sequences obtained were analysed and compared with the database http://www.mlst.net. The combination of the seven obtained alleles for each isolate, give us a specific sequence type (ST) and clonal complex (CC) (Homan et al., 2002).

VI.1.3.5. Protein extraction

Frozen *van*A-containing *Enterococcus* cell stocks were streaked onto Luria-Bertani (LB) plates and grown at 37°C. Single colonies of *van*A-containing *Enterococcus* strains were conducted in 250 mL of M9 minimal medium supplemented with 4 g/L of glucose in covered 1 L Erlenmeyer flasks at 37°C. Cells were harvested from the exponential phase in all experiments. The cells were pelleted down at 10,000 rpm at 4°C for 3 min. The pellet should be visible after spinning and resuspended in an equal volume of pre-warmed phosphate-buffered saline (PBS) pH 7.4 (Görg et al., 2004). After new centrifugation pellet was suspended in 0.2 mL of SDS sample solubilization buffer. The sample was sonicated with an ultrasonic homogenizer. The disrupted cells were centrifuged in an Eppendorf microfuge at maximum speed (14,000g) for 30 minutes at 4°C. For SDS-PAGE experiment the supernatant was collected and resuspended in an equal volume of buffer containing 0.5 M Tris HCl pH 8.0, glycerol, SDS and bromophenol blue.

VI.1.3.6. One-dimensional and coloration

One-dimensional electrophoresis was conducted on vertical gel with SDS-polyacrylamide gels (T= 12.52%, = 0.97%) in a Hoefer™SE 600 Ruby® (Amersham Biosciences) unit, following Laemmli (Laemmli, 1970) with some specific modifications (Igrejas, 2000). Electrophoresis was carried out with a constant current of 30 mA per gel until the dye-front reached the bottom of the gels which were stained with Coomassie Brilliant Blue R250 during 24 hours and washed in water overnight. It was then fixated in trichloroacetic acid 6% for four hours and in glycerol 5% for two hours (Görg et al., 2000). Reproducibility of the SDS-PAGE technique for enterococci characterization was confirmed by the analysis of triplicate protein extracts in which cells grown independently had similar banding patterns.

VI.1.3.7. Two-dimensional electrophoresis and proteomics

2-DE was performed according to the principles of O'Farrell (O'Farrell, 1975) but with IPG (Immobiline™pH Gradient) technology (Görg et al., 2007). Protein samples of *van*A *E. durans* isolate [SG 3 VRE] were used in parallel with those *van*A *E. durans* isolate [SG 41 VRE] proteins. For IEF, precast IPG strips with linear gradient of pH 4-7 were passively rehydrated overnight (12 to 16 hours) in a reswelling tray with rehydration buffer (8 M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte IPG buffer pH 3-10) at room temperature IPG strips were covered with DryStrip Cover Fluid (Plus One, Amersham Biosciences). Lyses buffer [9.5 M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3-10) and 10 mM Pefabloc® proteinase inhibitor] was added to the two *van*A-*containing* enterococci isolates (1:1). Samples containing a total of 100 µg of protein were loaded into 13 cm IPG strips (pH 4-7 NL, Amersham Biosciences, UK) (Görg et al., 2000). The sample solution was then applied in the previously rehydrated IPG strips pH 4-7 by cup loading and then proteins were focused sequentially at 500 V for 1 h, 1000 V for 1 h, 8000 V for 2 h 30 and finally 8000 V incremented to 12505 V/h on an Ettan™ IPGPhor II™ (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed according to Görg (Görg et al., 2007) and the GE Healthcare protocol for IPG strips pH 4-7 of 13 cm, in order to obtain the optimized running conditions, resulting in a final 5 h 25 hour run. Focused IPG strips were then stored at -80°C in plastic bags. Before running the second dimension, strips were equilibrated twice 15 minutes in equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer (pH 8.8)). In the first equilibration it was added 1% DTT to the original equilibration buffer and to the second 4% iodoacetamide, and also bromophenol blue was added to both solutions. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied onto a 12.52% polyacrylamide gels in a Hoefer™ SE 600 Ruby® (Amersham Biosciences) unit. Some modifications were introduced in the SDS-PAGE technique previously reported by Laemmli (Laemmli, 1970), that allowed its resolution to be increased, with proper insertion of the IPG strips in the stacking gel (Igrejas, 2000; Laemmli, 1970). After SDS-PAGE, the 2-DE gels were fixated in 40% methanol/10% acetic acid for one hour and afterwards stained overnight in Coomassie Brilliant Blue G-250 (Görg et al., 2004). Coomassie-stained gels were scanned on a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA), and the resulting digitized images were analyzed using Image Master 5.0 software (Amersham Biosciences; GE Healthcare).

VI.1.3.8. Protein identification by MALDI-TOF/TOF

Spots of expression in all gels were manually excised from the gels and analyzed using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). The gel pieces were washed three times with 25 mM ammonium bicabornate/50% ACN, one time

with ACN and dried in a SpeedVac (Thermo Savant). 25 mL of 10 mg/mL sequence grade modified porcine trypsin (Promega) in 25 mM ammonium bicabornate was added to the dried gel pieces and the samples were incubated overnight at 37°C. Extraction of tryptic peptides was performed by addition of 10% of formic acid (FA)/50% ACN three times being lyophilised in a SpeedVac (Thermo Savant). Tryptic peptides were ressuspended in 10 mL of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/0.1% formic acid. Aliquots of samples (0.5 μL) were spotted onto the MALDI sample target plate.

Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Mass spectra were internally calibrated with autodigest peaks of trypsin (MH+: 842.5, 2211.42 Da) allowing a mass accuracy of better than 25 ppm.

VI.1.3.9. Database search

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v 2.1.04, Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data. Swiss-Prot nonredundant protein sequence database was used for all searches under *Enterococcus*. Database search parameters as follows: carbamidomethylation and propionamide of cysteine (+71Da) as a variable modification as well as oxidation of methionine (+16Da), and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 25 ppm and fragment ion mass tolerance was 0.3 Da. Protein identifications were considered as reliable when the MASCOT score was > 70 (MASCOT score was calculated as $-10 \times \log P$, where P is the probability that the observed match is a random event.). This is the lowest score indicated by the program as significant ($P < 0.05$) and indicated by the probability of incorrect protein identification.

VI.1.3.10. Sequence alignments and construction of the phylogenetic tree

The analysis was performed on the Phylogeny.fr platform and comprised the following different steps. Sequences were aligned with MUSCLE (v3.7) configured for highest accuracy (MUSCLE with default settings). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). The JTT substitution model was selected assuming an estimated proportion of invariant sites (of 0.000) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was

estimated directly from the data (gamma = 12.476). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation of the phylogenetic tree (phenogram) was performed with Drawgram from the PHYLIP package (v3.66).

VI.1.4. Results

VI.1.4.1. Phenotypic and genetic characterization of enterococci isolates to antibiotic resistance

Table S3 shows the different antimicrobial resistance genotypes detected in the enterococci isolates showing resistance to one or more antibiotic agents recovered from seagulls. It demonstrates that the majority of the enterococci strains carried the combination *tet*(M)+*tet*(L) and *erm*(B) genes. Different genomic profiles (17) were demonstrated in all of the enterococci isolates. The most prevalent genotype included the *tet*(M)+*tet*(L)+Tn*916*+Tn*5397*+*erm*(B) genes.

*van*A-containing enterococcal strains were detected in six of the 57 seagull samples (10.5%). Four of the *van*A-containing *Enterococcus* strains were identified as *E. durans*, and two as *E. faecium*. The characteristics of these strains and of the animals from which they were recovered are shown in Table S4. All *van*A strains showed high level vancomycin (MIC ≥128 mg/L), and teicoplanin resistance (MIC 64 mg/L); most of them showed resistance for tetracycline (n=5), and all of them for erythromycin. The *tet*(M) gene was found in all five tetracycline-resistant *van*A strains, in most of the cases associated with *tet*(L) gene (Table S4); the *erm*(B) gene was demonstrated in all six erythromycin-resistant *van*A strains. Enterococci with intrinsic vancomycin resistance (*van*C-1 or *van*C-2/3 gene) were not found. The *esp* gene was not detected among our isolates. The *hyl* virulence gene was detected in both *van*A-containing *E. faecium* isolates in this study (Table S4), and they harboured the *pur*K1 allele, and in addition showed ampicillin and ciprofloxacin resistance. Additionally, the MLST typing of the two *van*A-positive *E. faecium* isolates demonstrated the ST5 type (included in CC17 clonal complex).

VI.1.4.2. One-dimensional electrophoresis

The enterococci isolates were displayed in the one-dimensional gel electrophoresis based on the genotypic profiles similarities. The SDS-PAGE of whole-cell extracts of the 6 *van*A-containing enterococci strains are shown in Figure VI.1.1. Analysis of different strains by SDS-PAGE gave reproducible whole-cell proteins patterns which allowed differentiation between the species included in this study (*Ed* for *E. durans* and *Ef* for *E. faecium*). The major differences between these two species were identified in the 97-45 kDa region. The four *E. durans* strains studied presented two different genomic patterns (*tet*(M)-*tet*(L)-*erm*(B) and *tet*(M)-*tet*(L)-*erm*(B)-*hyl*), were traduced into three different protein profiles. This picture reveals the higher complexity of the proteome when compared with the static genome screened by PCR. Similar antibiotic resistance had similar protein

profiles for *E. durans* (SG 1 and SG 2) (Figure V1.1.1, Lanes 2 and 3) but shows evident differences when compared strains SG3 and SG56, both identified as *E. durans* (Figure V1.1.1, Lanes 4 and 5). Although these two strains show the same genotypic pattern (*tet*(M)-*tet*(L)-*erm*(B)-*hyl*) presents very clear differences between protein bands (1-4 in contrast to 5-6). Differences revealed by SDS-PAGE for the two *E. faecium* strains characterised which have differences in tetracycline gene, SG 41 (*erm*(B)-*hyl*) and SG 50 (*tet*(M)-*tet*(L)-*erm*(B)-*hyl*) were represented by three different bands (7-9).

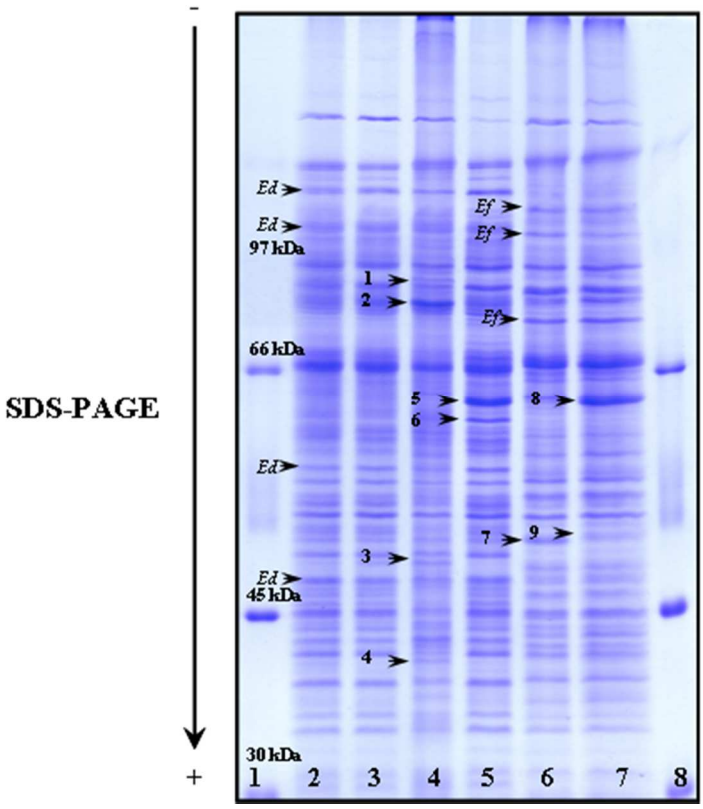

Figure VI.1.1 | SDS-PAGE of vancomycin-resistant enterococcal strains

SDS-PAGE of vancomycin-resistant enterococcal strains. Lanes 1 and 8: Molecular mass markers (LMW Pharmacia kit); Lanes 2 to 5: *E. durans* (Gv1, Gv2, Gv3 and Gv56, respectively); Lanes 6 and 7: *E. faecium* (Gv41 and Gv50, respectively).

VI.1.5. Two-dimensional Electrophoresis

A comparative analysis among the strains has been carried out. The protein expressions of the two vancomycin-containing enterococci (*van*A *E. faecium* SG 41 and *van*A *E. durans* SG 3) strains were visualized on 2-DE gels (Figures VI.1.2 and VI.1.3). The use of pH 4-7 IPG strips resulted in a well spread protein spots display which contributed to an accurate and safe excision and image identification of the spots. For each sample SG 3 and SG 41, a total of 60 relevant protein spots were collected for their analysis using MALDI-TOF mass spectrometry.

The peptide mass peaks were compared with those in the NCBI database http://www.ncbi.nlm.nih.gov/webcite, and the protein identification data including genebank ID, MW, PI value, mascot score, number of matched peptides and sequence coverage ratio (%) are listed in Table S5 and Table S6 for the *van*A *E. faecium* SG 41 proteins. The identified proteins were showing diverse functional activities including glycolysis, conjugation, translation, protein biosynthesis, among others (Figures VI.1.4 and VI.1.5). Replicate sequences, truncated sequences, and sequences with partial alignments were removed from the BLAST results (not shown). From the collected sequences were selected to represent the initial tree. These sequences were aligned, and a phylogenetic tree was constructed by the minimum-evolution method to root the tree. The clustering of the initial phylogenetic tree indicated that all of the proteins included in the data set diverged from a common ancestor (Figure V1.1.6).

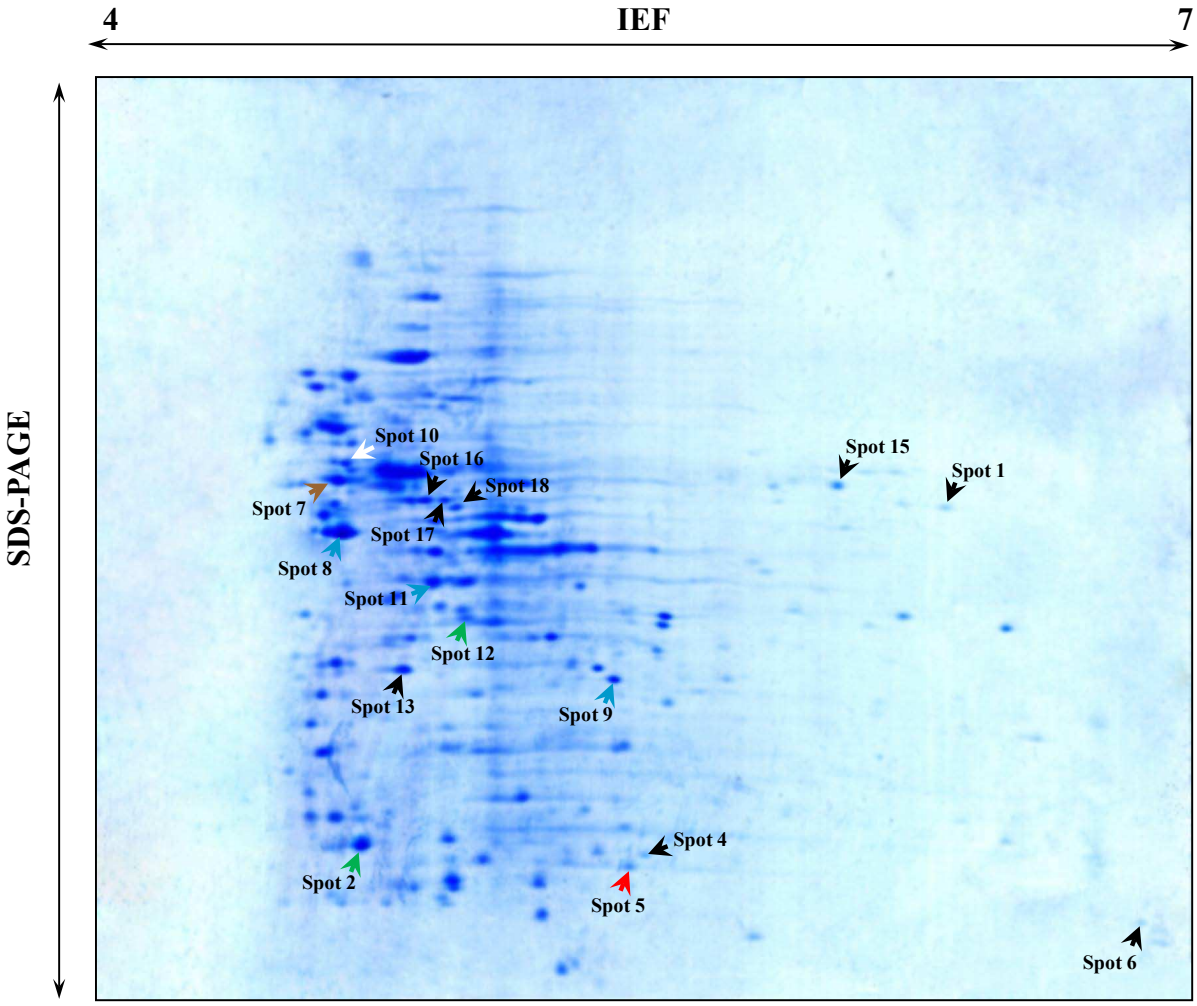

Figure VI.1.2 | 2-DE gel image of SG 3 VRE with IPG strips pH4-7

Green: Protein biosynthesis; Yellow: ATP synthesis; Blue: Glycolysis; Brown: Conjugation; Red: Antibiotic resistance; Black: Proteins of *van*A *E. durans* SG 3 isolate with different biological processes from the proteins of *van*A *E. faecium* SG 41 isolate

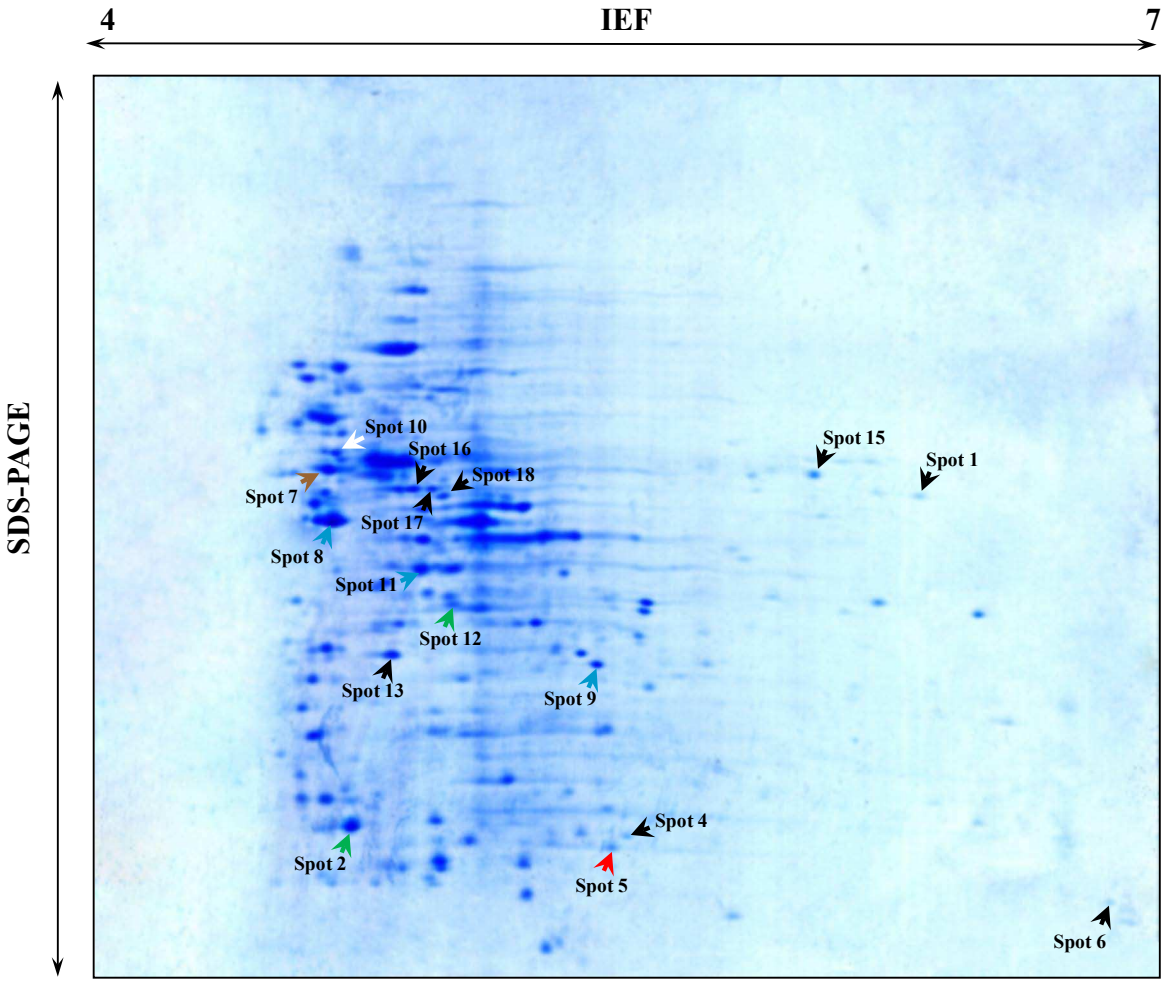

Figure VI.1.3 | 2-DE gel image of SG 41 VRE with IPG strips pH4-7

Green: Protein biosynthesis; Blue: Glycolysis; Brown: Conjugation; Red: Antibiotic resistance; Black: Proteins of *van*A *E. faecium* SG 41 isolate with different biological processes from the proteins of *van*A *E. durans* SG 3 isolate

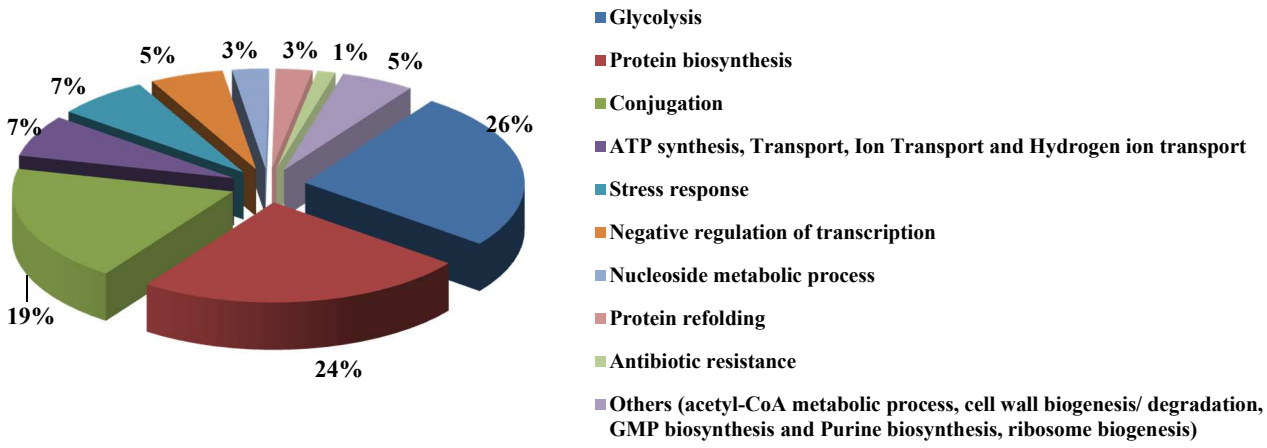

Figure VI.1.4 | Distribution of the biological processes related to the protein spots found in the 2-DE gel of the *van*A *E. durans* SG 3 isolate

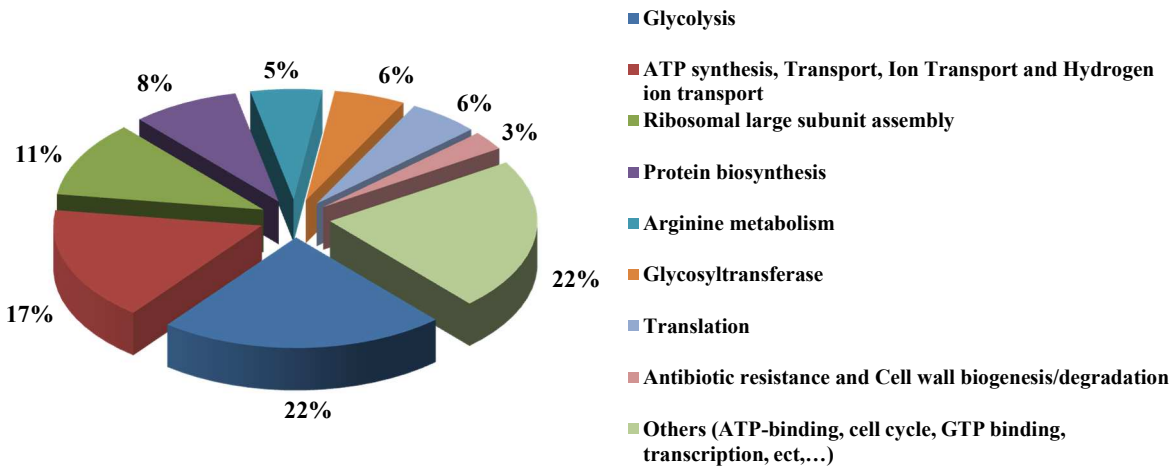

Figure VI.1.5 | Distribution of the biological processes related to the protein spots found in the 2-DE gel of the *van*A *E. faecium* SG 41 isolate

VI.1.6. Discussion

Unlike the genome, the proteome is dynamic: it varies according to the cell type and the functional state of the cell. In addition, the proteome shows characteristic perturbations in response to disease and external stimuli (Marshall & Williams, 2002). Nevertheless three different bands were observed (7 until 9) which can represent hypothetic correlation with differences in genomic data. The molecular weight of these bands is similar to those obtained from the 2-DE separations, which identify nine proteins associated with resistance to tetracycline (Xu et al., 2006). The *esp* gene, that encodes an enterococcal surface protein associated with the ability to biofilm formation on abiotic surfaces (Eaton & Gasson, 2001), is more frequently found in clinical isolates (Shankar et al., 1999), and this fact could explain the absence of detection among the commensal isolates analysed in this study. The wide dissemination of vancomycin-resistant *E. faecium* isolates of the epidemic-virulent clonal complex-17 (CC17), which harbour the *pur*K1 allele, and are very frequently associated with ampicillin and ciprofloxacin resistance, and also with the presence of *esp* and *hyl* virulence genes has been reported (Gilmore et al., 2002; Huycke et al., 1998; Klare et al., 2005; Leavis et al., 2006). Two of our isolates present most of these characteristics (although do not harbour *esp* gene).

In our study of the identified proteins, it is important to point out the presence of vancomycin/teicoplanin A-type resistance protein *van*A in *van*A-*E. durans* SG 3 isolate. It has therefore been postulated that resistant cells produce peptidoglycan precursors that terminate in the depsipeptide D-alanine-2-D-hydroxy acid rather than the dipeptide D-alanine-D-alanine, thus preventing vancomycin binding (Arthur et al., 1992b). Vancomycin-dependence results from a mutation that inactivates the D-Ala: D-Ala ligase gene (*ddl*) in the chromosome, so that the mutant strain no longer produces D-Ala: D-Ala-ending peptidoglycan precursors. Thus, cell wall synthesis in the mutant strain is dependent on the production of alternative peptidoglycan precursors

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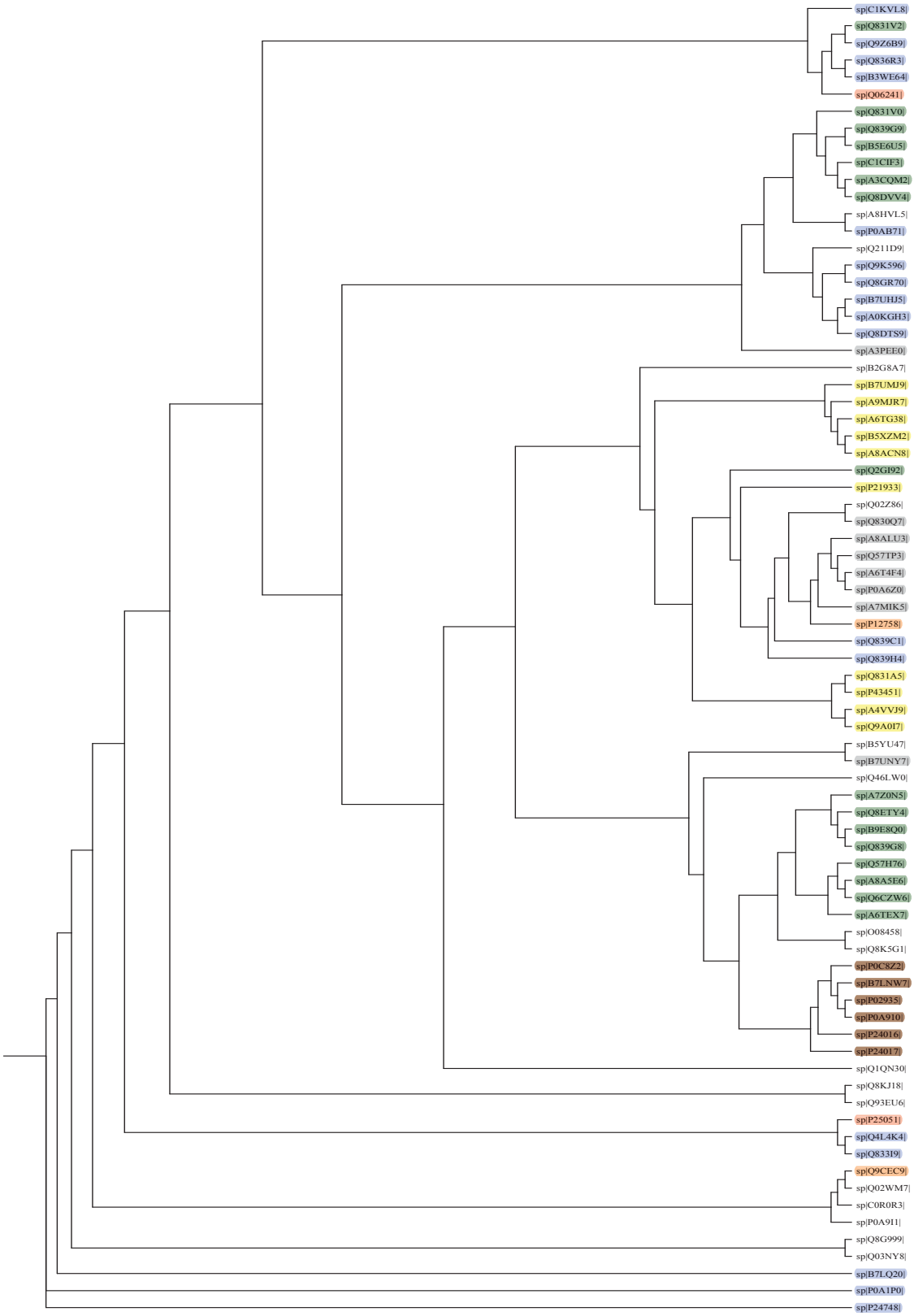

Figure VI.1.6 | Phylogenetic tree of FASTA protein sequences of all proteins identified

The full alignment of these sequences were done with MUSCLE (v3.7) configured for highest accuracy. Legend: Green: Protein biosynthesis; Yellow: ATP synthesis; Blue: Glycolysis; Brown: Conjugation; Red: Antibiotic resistance; Black: Proteins of *van*A *E. durans* SG 3 isolate with different biological processes from the proteins of *van*A strains

The D-Ala: D-Lac ligase activity of *van*A and *van*B can replace ddl activity by production of D-Ala: D-Lac-ending peptidoglycan precursors instead of the native D-Ala: D-Ala-ending precursors. As both resistances are inducible with vancomycin, the production of alternate precursors requires the presence of vancomycin and the mutant strain becomes vancomycin-dependent for growth (Kak & Chow, 2002). It is interesting to find the ddl protein in *van*A *E. durans* isolate from faecal sample of seagulls because since *E. faecalis* and *E. faecium* represent more than 95% of the clinical isolates collected, identification of enterococci based on the amplification of a fragment internal to the *ddl* gene encoding a D-Ala-D-Ala ligase included only these two species (Cetinkaya et al., 2000).

In *van*A *E. faecium* SG 41 isolate it is notice to indicate the presence of D-alanyl-D-alanine dipeptidase that hydrolyzes D-Ala-D-Ala, thereby preventing vancomycin binding. The unstability of depsipeptide could also make VanY, a D-, D-carboxypeptidase in the *van* gene cluster which could participate to vancomycin resistance by removing D-Ala residue from C-terminus of peptidoglycan (Arthur et al., 1992a), functionally unnecessary for high-level vancomycin resistance in *E. faecium* isolate.

From a total of 60 protein spots identified in *van*A *E. durans* SG 3 isolate, 5 proteins were found as related to stress response. Chaperone protein dnaK was detected and shows to be involved in the stress response mechanism for heat, a very important reaction for the survival of bacteria such as enterococci and that contributes for the antibiotic resistance capability (McClelland et al., 2005). The protein dnaK was detected in spot 20 as linked to two *E. coli* serotype 0157:H7 strains (accession number P0A6Z0 and A6T4F4), one *Citrobacter koseri* strain (A8ALU3), one *Enterobacter sakazakii* strain (A7MIK5) and one *Salmonella choleraesuis* strain (Q57TP3). In the spot 21 was found the protein 60 kDa chaperonin (groL) in the *van*A *E. durans* SG 3 isolate related to *Enterococcus faecalis* (Q93EU6) and *Streptococcus constellatus* (Q8KJ18). The protein groL was also present in the spot 10 of the *van*A *E. faecium* SG 41 isolate. This protein prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions (Laport et al., 2004; Paulsen et al., 2003).

The D-alanine-D-alanine ligase protein (A3PEE0) was also found in this isolate (SG 41) as being related with *Prochlorococcus marinus*, where it is involved in the cell wall biogenesis and peptidoglycan biosynthesis (Makarova et al., 2006). It is important to highlight the presence in the *van*A *E. durans* SG 3 isolate of the wrbA flavoprotein (B7UNY7 and B5YU47) related to two enterhemorrhagic *E. coli* strains (*Escherichia coli* O127:H6 and *Escherichia coli* O157:H7, respectively). WrbA (tryptophan [W] repressor-binding protein) was discovered in *Escherichia coli*, where it was proposed to play a role in regulation of the tryptophan operon. This protein seems to improve the formation and/or stability of noncovalent complexes between the trp repressor protein and operator-bearing DNA (Iguchi et al., 2009). This wrbA flavoprotein was also detected in *E. coli* C580 isolated from faecal sample of human by our investigation group and shows the partage of different sequences among different bacteria (Pinto, 2010).

Our results clearly show that electrophoretic methods can provide valuable epidemiological information that may be used to isolate and characterize *Enterococcus* spp. The results are in accordance with previous results of Wang et al. (Wang et al., 2010) that demonstrated that many proteins involved in antibiotic resistance were differentially regulated by vancomycin which also triggered innate signal regulators, adhesion factors, and metabolic gene expression in *E. faecalis*. Therefore, these responses may enable *Enterococcus* spp. to adapt, survive, and remain pathogenic even under pressure of vancomycin treatment.

VI.1.7. Supplementary material

Supplementary material (Tables S3, S4, S5 and S6) regarding this manuscript is available at Proteome Sci online (http://www.proteomesci.com/content/8/1/48).

VI.1.8. Conclusion

This work, albeit preliminary in nature, reveals the complexity of expressed proteins in bacteria or different species and profiles of antibiotic resistance. SDS-PAGE patterns can be obtained easily and rapidly, are reproducible and do not require any sophisticated equipment and expensive reagents. Although protein profiles also represent phenotypic characteristics, they are considered to provide an excellent approximation of a microorganism's genome information. In addition, their extractability, sequence homology, and post-translational modifications (PTMs) make proteomic analysis complex and informative. Proteomic methodologies contribute towards determining antimicrobial resistance mechanism(s) through the capacity to analysis global changes of bacteria. The totality of proteins identified in the present work are not necessarily related with antibiotics, hence the importance of 2-DE. Epidemiological studies in different animals should be continued in the future to elucidate the evolution of *van*A enterococcal and enterococci strains in different ecosystems. The complete sequencing and comparative proteome of some of these strains were isolated for the first time in this wild population of seagulls as well as the recognition of these proteins as markers in antibiotic resistance mechanisms. This biochemical and genomic foundation coupled to the parallel improvements of proteomic procedures enabled us to study *van*A-enterococci proteome. This now provides a sound basis for a comprehensive understanding of adaptability to environment and pathogenicity mechanisms. This work reports the impact of proteomics on our knowledge of *van*A enterococci strains.

To our knowledge, this study is the first report which identifies candidate proteins related in antibiotic resistance and involved in the general stress response in *van*A-containing *Enterococcus faecium* and *durans* species. Therefore, our results may reflect the expression of a few membrane

proteins involved in antibiotic resistance. Correlation with web databases allowed the exact identification and characterization of the proteins present as well as their functions and relations within known biological processes occurring at the cellular level in enterococci. Proteomics and protein identification by 2-DE correlated with MALDI/TOF-TOF and bioinformatic databases are expected to become increasingly essential in elucidating the mechanisms of antibiotic resistance.

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VI.2. Comparative proteomic map among *van*A-containing *Enterococcus* isolated from yellow-legged gulls

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VI.2.1. Abstract

The increase of vancomycin-resistant enterococci (VRE) represents a threat for patient care and creates a reservoir of mobile resistance genes for other, more virulent pathogens. The existence of VRE in different ecologic niches complicates the understanding of its epidemiology. The aim of the present study was to study the proteome of 2 *van*A strains recovered from seagull faecal samples. The *van*A *E. durans* and *van*A *E. faecium* isolates presented different genomic patterns: *tet*(M)-*tet*(L)-*erm*(B) and *tet*(M)-*tet*(L)-*erm*(B)-*hyl*, respectively. A total of 123 spots were excised from two-dimensional gel electrophoresis (2-DE) gel of *van*A *E. durans* SG 2 strain, and 16 were successfully identified by MS, representing 42 different proteins. For the *van*A *E. faecium* SG 50 strain, 93 spots were excised from the 2-DE gel and 23 were identified, representing 47 different proteins. The vancomycin/teicoplanin A-type resistance protein *van*A in *E. durans* SG 2 strain was present in two different spots. The identified proteins have shown diverse functional activities including glycolysis, conjugation, translation, protein biosynthesis, among others. This work reports the impact of proteomics on knowledge of *van*A-containing enterococci strains and will be helpful to further understand the expression of proteins in antibiotic-resistant bacteria.

VI.2.2. Introduction

Proteome analysis is a tool that can be used to visualize and, also, to compare complex mixtures of proteins, and to acquire important information about the individual proteins concerned in specific biological responses (Patterson & Aebersold, 2003). Proteomic is defined as the expressed complement of a genome. In comparison to the genome, the proteome is used to define a series of proteins expressed by a certain organism, under certain conditions, serving as unique and informative information of both its phenotypic state. This results in cell responses to physiological and environmental perturbations, and genomic information reflected in the amino acid sequences of expressed proteins. Therefore, the principal concern of proteomic resides, in the identification of proteins related to, in particular, cellular processes or presenting altered expression profiles as a consequence of different physiological conditions (Fish & Ohlinger, 2006).

Various analytical tools such as two dimensional gel electrophoresis, mass spectrometry and searches in generalist and Expressed Sequence Tags (EST) databases, modified the protein identification process (Jungblut et al., 2008). Although not usually used, proteomic approaches participate in determining antimicrobial resistance mechanism(s) and other cell metabolic alterations through the ability to study overall changes in bacteria (Cash et al., 1999). The model of protein species linked to the antimicrobial resistance has been investigated in a diversity of microorganisms and with different antimicrobial agents (Andrade et al., 2008; Cash et al., 1999; Yoo et al., 2007). The evaluation of changes in protein profiles in response to various mechanisms of stress, such as the susceptibility to antimicrobial agents or the modifications related to antibiotic resistance could represent an integrating method for the development of new therapeutic treatment and antimicrobial agents. Bacterial surface proteins are important for the host pathogen interaction and they are commonly implicated in disease pathogenesis (Cash et al., 1999).

Enterococcus spp. live as commensals of the gastrointestinal tract of warm-blooded animals, being the most abundant Gram-positive cocci in humans and in animals (Tannock & Cook, 2002). Recently, the incidence of nosocomial enterococal infections has increased distinctly (Udo et al., 2003). This genus is also recognized as important opportunistic pathogens, and reveals intrinsic resistance to a number of antimicrobial agents, in addition to the acquired multidrug resistance (Coque et al., 1995).

The increase of vancomycin-resistant enterococci (VRE) causes several challenges. Firstly, most of VRE are frequently also resistant to other available drugs e.g., aminoglycosides or ampicillin. Secondly, there is the possibility that the vancomycin resistance genes present in VRE could be transferred to other Gram-positive microorganisms. In addition to the currently common detection of multiresistant bacteria in areas with high human density (Cole et al., 2005) their emergence in more remote areas like high mountain regions or natural reserve is even more alarming (Dolejska et al., 2007; Pinto et al., 2011; Radhouani et al., 2010b). Monitoring the prevalence

of resistance in indicator bacteria such as vancomycin-resistant enterococci in different populations, animals, patients and healthy humans, makes it feasible to compare the prevalence of resistance and to detect the transfer of resistant bacteria or resistance genes from animals to humans and vice versa (Martel et al., 2001). Microbial resistance to antibiotics is a worldwide problem in human and veterinary medicine. Commonly, it is usual that the main risk factor for the increase of this situation is an extensive use of antibiotics that leads to the dissemination of resistant bacteria and resistance genes in animals and humans (van den Bogaard & Stobberingh, 2000).

The wild birds seem to represent a significant reservoir, or at least source of *van*A-enterococcal strains. Consequently, this may represent a significant hazard to human and animal health by transmitting these strains into waterways and other environmental sources via their faecal deposits. Although wild birds rarely come into contact with antimicrobial agents, arguing against the existence of direct selective pressure on birds, nevertheless they can be infected or colonized by resistant bacteria. Water contact and acquisition via food seem to be major aspects of transmission of the resistant bacteria of human or veterinary origin to wild animals (Cole et al., 2005; Kozak et al., 2009). Wild birds or wild animals in general could, therefore, serve as reservoirs of resistant bacteria and genetic determinants of the antimicrobial resistance (Dolejska et al., 2007).

In the present study, we examined the proteome of 2 *van*A strains recovered from seagull faecal samples. This evaluation was carried out in order to compare the proteins obtained from these strains with the results obtained in a previous published study using a different group of strains (Radhouani et al., 2010b). The combination of two high-resolution methods, isoelectric focusing and SDS polyacrylamide gel electrophoresis permitted the separation of numerous proteins of *van*A-containing *Enterococcus* isolates and highlight the presence of vancomycin/teicoplanin A-type resistance protein vanA.

VI.2.3. Material and methods

VI.2.3.1. Samples and bacteria

The phenotypic and genetic profiles of two *van*A containing *Enterococcus* strains (*van*A *E. durans* SG2 and *van*A *E. faecium* SG50) as well as the SDS-PAGE of whole cell extracts of them were studied in a previous report (Radhouani et al., 2010b). The complete proteomic analysis of these two *van*A containing strains SG2 and SG50 has been the objective of the present study. These strains were previously obtained from faecal samples of yellow-legged seagulls, randomly recovered in the beaches of Berlengas Islands National Reserve of Portugal (Radhouani et al., 2010b).

VI.2.3.2. Virulence factor genes

The presence of genes encoding different virulence factors (*gel*E, *fsr*, *ace, cpd*, *agg and cyl*L_LL_SABM) was verified by PCR using primers and conditions were previously described (Eaton & Gasson, 2001; Pillai et al., 2002; Semedo et al., 2003). Positive and negative controls obtained from the collection of the University of Trás-os-Montes and Alto Douro (Portugal) were included in all assays. The presence of *hyl* or *esp* gene in these strains was previously reported (Radhouani et al., 2010b).

VI.2.3.3. Assay of gelatinase activity

Gelatinase production was detected by inoculating the enterococci onto freshly prepared tryptic soy agar plates (Difco; 236950, Le Pont de Claix, France) containing 1.5% of skim milk (Difco; 232100). Plates were incubated overnight at 37°C and then cooled to ambient temperature for 2 h. The appearance of a transparent halo around the colonies was considered to be a positive indication of gelatinase production (Jett et al., 1994).

VI.2.3.4. Assay of haemolytic assay

The production of hemolysin was determined by streaking bacterial cultures, grown overnight at 37°C in brain heart infusion agar (Difco; 241830), on columbia agar plates supplemented with 5% of horse blood (BioMérieux; 43050, La Balme, Les Grottes, France). Plates were incubated at 37°C for 72h in aerobic conditions and after that the plates were examined for haemolysis. The haemolytic reaction was recorded by the observation of a clear zone of hydrolysis around the colonies (β-haemolysis), a partial hydrolysis (α-haemolysis) and a nonreaction (γ-haemolysis). When observed, greenish zones around the colonies were interpreted as α-haemolysis and taken as negative for the assessment of β-haemolytic activity (Semedo et al., 2003).

VI.2.3.5. PCR amplification of pbp5 gene

Total DNA was extracted from *van*A *E. faecium* SG 50 isolate by the InstaGene Matrix (Bio-Rad; 732-6030, Hercules, United States), and 10µl of DNA was used for the PCR reaction, the $MgCl_2$ concentration being of 3.5 mM. The primers used for PCR amplification of *pbp*5 gene were the following ones: F 5'-AACAAAATGACAAACGGG-3'; R 5'-TATCCTTGGTTATCAGGG-3'. PCR conditions were as follows: 95°C initially for 15 min; 94°C for 30 s, 54°C for 30 s, 72°C for 2 min over 30 cycles followed by a final 7 min extension period at 72°C (Jureen et al., 2003).

VI.2.3.6. DNA sequence analysis

The *pbp5* PCR products were purified with the QiaQuick PCR purification kit (Qiagen Inc.) according to the instructions of the manufacturer. The purified products were sequenced in both

strands on the ABI Prism 3700 DNA sequencer (Perkin-Elmer). The obtained sequences were compared through bioinformatics tools to that of *pbp*5 included in GenBank accession no. X84860.

VI.2.3.7. Protein extraction

Frozen *van*A-containing *Enterococcus* cell stocks were streaked onto Luria-Bertani (LB) plates and grown at 37ºC. Single colonies of *van*A-containing *Enterococcus* strains were conducted in 250 mL of M9 minimal medium supplemented with 4 g/L of glucose in covered 1 L Erlenmeyer flasks at 37ºC. Cells were harvested from the exponential phase in all experiments. The cells were pelleted down at 10,000 rpm at 4ºC for 3 min. The pellet was supposed to be visible after spinning and resuspended in an equal volume of pre-warmed phosphate-buffered saline (PBS) pH 7.4 (Görg et al., 2004). After new centrifugation pellet was suspended in 0.2 mL of SDS sample solubilization buffer. The sample was sonicated with an ultrasonic homogenizer (6×10 s, 4°C at 100 W). The disrupted cells were centrifuged in an Eppendorf microfuge at maximum speed (14,000 rpm) for 30 minutes at 4°C. For SDS-PAGE experiment the supernatant was collected and resuspended in an equal volume of buffer containing 0.5 M Tris HCl pH 8.0, glycerol, SDS and bromophenol blue.

VI.2.3.8. Two-dimensional electrophoresis and proteomics

The 2-DE was performed according to the principles of O'Farrell (O'Farrell, 1975) but with IPG (Immobiline™ pH Gradient) technology (Görg et al., 2007). Protein samples of *van*A *E. durans* isolate (SG 2 VRE) were used in parallel with those of *van*A *E. faecium* isolate (SG 50 VRE) proteins. For IEF, precast IPG strips with linear gradient of pH 4-7 were passively rehydrated overnight (12 to 16 hours) in a reswelling tray with rehydration buffer (8M urea, 1% CHAPS, 0.4% DTT, 0.5% carrier ampholyte IPG buffer pH 3-10) at room temperature and IPG strips were covered with DryStrip Cover Fluid (Plus One, Amersham Biosciences). Lyses buffer [9.5M urea, 1% (w/v) DTT, 2% (w/v) CHAPS, 2% (v/v) carrier ampholytes (pH 3-10) and 10 mM Pefabloc® proteinase inhibitor] was added to the two *van*A-*containing* enterococci isolates (1:1). Samples containing a total of 73.5 µg of protein were loaded into 13 cm IPG strips (pH 4–7 NL, Amersham Biosciences, UK) (Görg et al., 2000). The sample solution was then applied to the previously rehydrated IPG strips pH 4-7 by cup loading and then proteins were focused sequentially at 500 V for 1 h, 1000 V for 2 h, 8000 V for 2 h, 1000 V for 4h and, finally, 1000 V for 55min incremented to 23208 V/h on an Ettan™ IPGPhor II™ (Amersham Biosciences, Uppsala, Sweden). Seven IEF replicate runs were performed according to Görg (Görg et al., 2007) and the GE Healthcare protocol for IPG strips pH 4-7 of 13 cm, in order to obtain the optimized running conditions, resulting in a final around 10 hours run. Focused IPG strips were then stored at -80ºC in plastic bags. Before running the second dimension, strips were equilibrated twice for 15 minutes in an equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer (pH 8.8)). In the first equilibration, 1%

DTT was added to the original equilibration buffer and 4 % iodoacetamide to the second one, and, also, bromophenol blue was added to both solutions. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer, and then applied onto a 12.52% polyacrylamide gels in a Hoefer™ SE 600 Ruby® (Amersham Biosciences) unit. Some modifications were introduced in the SDS-PAGE technique previously reported by Laemmli (Laemmli, 1970), that allowed its resolution to be increased with a proper insertion of the IPG strips in the stacking gel (Igrejas, 2000; Laemmli, 1970). After SDS-PAGE, the 2-DE gels were fixated on 40% methanol/10% acetic acid for one hour and, afterwards, stained overnight in Coomassie Brilliant Blue G-250 (Görg et al., 2004). Coomassie-stained gels were scanned with a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA), and the resulting digitized images were analyzed using Image Master 5.0 software (Amersham Biosciences; GE Healthcare).

VI.2.3.9. Protein identification by MALDI-TOF/TOF

Spots of expression in all gels were manually excised from the gels and analyzed using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). The gel pieces were washed three times with 25mM ammonium bicabornate/50 % ACN, one time with ACN and dried in a SpeedVac (Thermo Savant). 25 mL of 10 mg/mL sequence grade modified porcine trypsin (Promega) in 25mM ammonium bicabornate was added to the dried gel pieces and the samples were incubated overnight at 37ºC. The extraction of tryptic peptides was performed by adding 10% of formic acid (FA)/50% ACN three times and being lyophilised in a SpeedVac (Thermo Savant). Tryptic peptides were ressuspended in 10 mL of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/ 0.1% formic acid. Aliquots of samples (0.5μL) were spotted onto the MALDI sample target plate. Peptide mass spectra were obtained through or from a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode.

Spectra were obtained in the mass range between 800 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Mass spectra were internally calibrated with autodigest peaks of trypsin (MH+: 842.5, 2211.42 Da) allowing a mass accuracy of better than 25 ppm.

VI.2.3.10. Database search

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v 2.1.04, Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data. Swiss-Prot nonredundant protein sequence

database was used for all searches under *Enterococcus*. Database search parameters are as follows: carbamidomethylation and propionamide of cysteine (+71Da) as a variable modification as well as oxidation of methionine (+16Da), and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 25 ppm and the fragment ion mass tolerance was 0.3 Da. Protein identifications were considered as reliable when the MASCOT score was > 70 (MASCOT score was calculated as $-10 \times \log P$, where P is the probability that the observed match is a random event). This is the lowest score indicated by the program as significant ($P < 0.05$) and indicated by the probability of incorrect protein identification.

VI.2.3.11. Sequence alignments and construction of the phylogenetic tree

The analysis was performed on the Phylogeny.fr platform and comprised the following steps. Sequences were aligned with MUSCLE (v3.7) configured for the highest accuracy (MUSCLE with default settings). After the alignment, the positions with gap were removed from the alignment. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.000) and 4 gamma-distributed rate categories to account for the rate heterogeneity across sites. The gamma shape parameter was fixed (alpha=12.4). The reliability for internal branch was assessed using the aLRT test (SH-Like). The graphical representation of the phylogenetic tree (phenogram) was performed with Drawgram from the PHYLIP package (v3.66) (Dereeper et al., 2008).

VI.2.4. Results

VI.2.4.1. Characteristics of the 2 vanA strains included in the study

The 2 *van*A *Enterococcus* strains showed high level of vancomycin (MIC ≥128 mg/L), and teicoplanin resistance (MIC ≥64 mg/L). The phenotype and genotype of antibiotic resistance of the two *van*A *Enterococcus* strains included in this study were previously reported (Radhouani et al., 2010b). Both strains showed high level of vancomycin and teicoplanin resistance, as well as resistance to tetracycline and erythromycin. *E. faecium* SG 50 isolate showed also resistance ciprofloxacin and ampicillin. The *van*A *E. durans* and *van*A *E. faecium* isolates presented different genomic patterns: *tet*(M)-*tet*(L)-*erm*(B) and *tet*(M)-*tet*(L)-*erm*(B)-*hyl*, respectively (Radhouani et al., 2010b).

Genes encoding virulence factors were studied in the two *van*A-containing *Enterococcus* isolates (SG2 and SG50). The *cpd* gene was detected in both *van*A enterococci. The *cyl*L_L gene was identified in the *van*A *E. durans* SG 2 srain. The two *van*A strains expressed gelatinase activity and carried the *gel*E gene. No beta-haemolytic activity was identified in both strains, although they showed alfa-hemolysis.

The sequence of the C-terminal region of *pbp*5 was analyzed in our *van*A *E. faecium* SG 50 isolate that showed ampicillin-resistance. Our isolate presented 11 amino acid substitutions in PBP5 protein (408Q → H, 427I → M, 470H → Q, 485M → A, 496N → K, 497F → I, 499A → T, 525E → D, 586V → L, 629E → V, 634N → Q), in relation with the reference one.

VI.2.4.2. Two-dimensional Electrophoresis

The SDS-PAGE of whole-cell extracts of the 2 *van*A-containing enterococci strains are shown in a previous study (Radhouani et al., 2010b). In the present study, a comparative analysis among the strains has been carried out. The protein expressions of the two vancomycin-containing enterococci (*van*A *E. durans* SG 2 and *van*A *E. faecium* SG 50) strains were analysed. *E. faecium* SG 50 was presented on 2-DE gel (Figure VI.2.1). The use of pH 4-7 IPG strips resulted in a well spread protein spots display which contributed to an accurate and safe excision and image identification of the spots. For each sample SG 2 and SG 50, a total of 123 and 93 relevant protein spots, respectively, were collected for analysis using MALDI-TOF mass spectrometry (Figure VI.2.2). These spots are consistently and systematically present in all replicates. The peptide mass peaks were compared with those in the NCBI database (http://www.ncbi.nlm.nih.gov/). The protein identification data including Genebank ID, MW, PI value, mascot score, number of matched peptides and sequence coverage ratio (%) are listed in Table S7 for and *van*A *E. durans* SG 2 and Table S8 for the *van*A *E. faecium* SG 50 proteins. The identified proteins were showing diverse functional activities, including glycolysis, conjugation, translation, protein biosynthesis, among others. Replicate sequences, truncated sequences, and sequences with partial alignments were removed from the BLAST results (not shown). From the collected sequences were selected to represent the initial tree. These sequences were aligned and a phylogenetic tree was constructed by using the minimum-evolution method to root the tree. The clustering of the initial phylogenetic tree indicated that all of the proteins included in the data set diverged from a common ancestor (Figure VI.2.3).

VI.2.5. Discussion

The worldwide appearance of antibiotic-resistant bacteria causes a severe threat to human health. Furthermore, it can add to the difficulties in controlling infectious diseases, the phenotype of resistance can generate metabolic changes that, in turn, can interfere with host-pathogen interactions. The commensal bacteria such as enterococci are generally considered pathogenic only if they carry human virulence factors (Eaton & Gasson, 2001). The two *van*A-containing *Enterococcus* strains included in the study were previously characterized for antibiotic resistance.

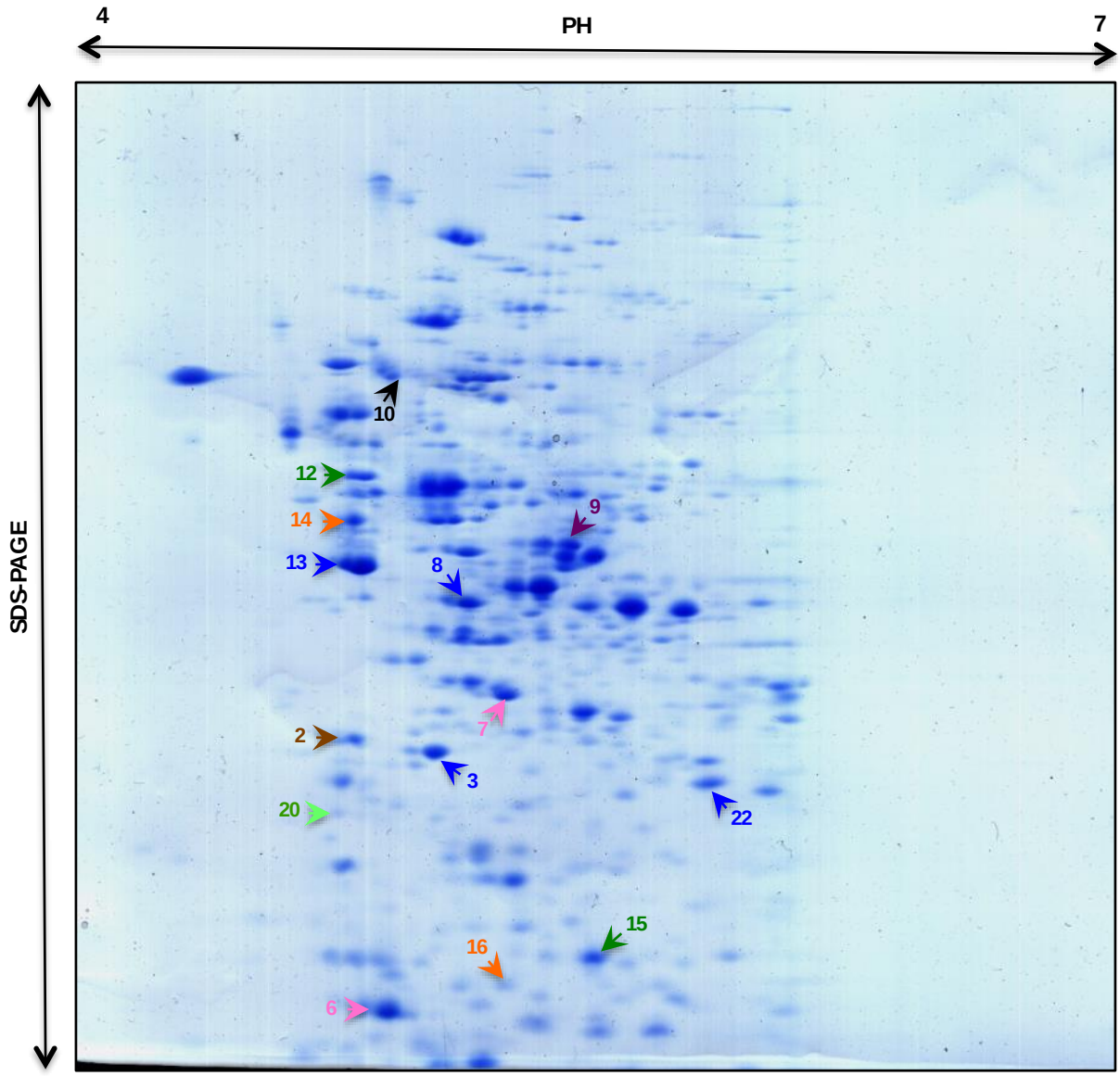

Figure VI.2.1 | 2-DE gel image of SG 50 VRE with IPG strips pH4-7

Arrows indicates peptides that are part of one or more proteins whose have a role in such pathways: Blue: Glycolysis, Orange: Stress response, Pink: Protein folding/ Protein biosynthesis, Brown: Pyrimidine biosynthesis, Bright green: Transcription, Light green: ATP synthesis, Violet: Arginine metabolism, Black: Phosphotransferase system

In our study, the *cpd* (a sex pheromone determinant) gene was detected in both *van*A strains (*E. durans* and *E. faecium*), which is similar to the results reported by others (Silva et al., 2011). The isolates with sex-pheromone determinants have the potential to acquire the respective sex-pheromone plasmids and, hence, the associated virulence and resistance determinants. So, the sex pheromone production may promote the acquisition of vancomycin resistance and other linked traits from *E. faecium* strains and lead to an increased virulence (Eaton & Gasson, 2001).

Gelatinase, encoded by the *gel*E gene, is an extracellular zinc endopeptidase that hydrolyses collagen, gelatin, hemoglobin and other bioactive compounds, and it has been shown to exacerbate endocarditis in an animal model, although this activity is not required) for pathogenesis (Jones & Deshpande, 2003).

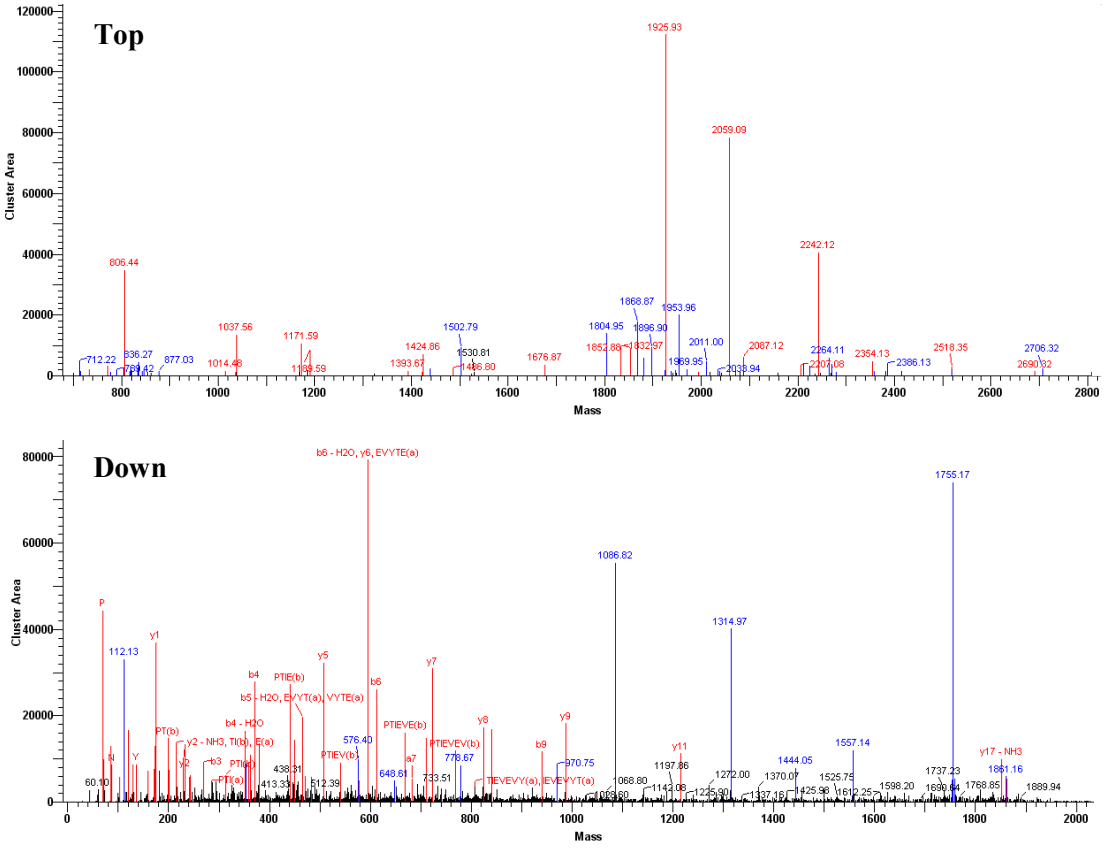

Figure VI.2.2 | MALDI/MS spectra obtained for Enolase from *van*A *E. durans* SG 2 strain

Top - MS analysis of tryptic peptides. Peaks in red matching enolase
Down - MALDI-TOF-TOF MS tandem spectrum of tryptic peptide [M+H]+; m/z 1925.93 identified as enolase. The identified sequence is GNPTIEVEVYTESGAFGR. On spectrum, peaks in red correspond to y and b series

Usually, the *gel*E gene has been detected with higher frequency suggesting that this virulence determinant is a common trait in the genus *Enterococcus*. In our study, the *gel*E was identified in both *van*A strains. Same results were observed in other reports (Radhouani et al., 2010a; Silva et al., 2011). The α-haemolysis could be a result of oxidation and a consequent lysis of the erythrocytes due to factors other than the production of enterococcal hemolysin (Semedo et al., 2003); therefore, the incubation under anaerobic conditions seems to be more reliable.

In this work, we performed a proteome analysis of two *van*A strains (*van*A *E. durans* SG 2 and *van*A *E. faecium* SG 50). A total of 123 spots were excised from 2-DE gel of SG 2 and 16 were successfully identified by MS, representing 42 different proteins. For the SG 50 strain, 93 spots were excised from the 2-DE gel and 23 were identified, representing 47 different proteins. It is important to highlight the presence of vancomycin/teicoplanin A-type resistance protein vanA in *van*A-*E. durans* SG 2 strain, in two different spots (12 and 14). VanA is a protein capable of utilizing both hydroxyl acids and D-Ala as substrates with a concomitant switch from ester to peptide bond formation dependent on pH (Park et al., 1996). Vancomycin inhibits the extracellular steps of peptidoglycan synthesis by binding it to the C-terminal D-alanyl-D-alanine (DAla- D-Ala) residues of cell wall precursors in enterococci (Roper et al., 2000).

The D-Ala-D-Ala target residues are synthesized intracellularly as a dipeptide by a D-Ala:D-Ala ligase. Then, they are added to UDP-Nacetylmuramyl- L-Ala-g-D-Glu-L-Lys (UDP-MurNac-tripeptide) by using an adding enzyme (Evers & Courvalin, 1996). The vancomycin/teicoplanin A-type resistance protein was also identified in *van*A-*E. durans* SG 3 isolate (Radhouani et al., 2010b).

The presence of vancomycin/teicoplanin A-type resistance protein in vancomycin-resistant enterococci in natural environments constitutes a serious impact on animal and human health. The emergence of vancomycin-resistant enterococci of human and veterinary origin (Grobbel et al., 2007) is inevitably accompanied by the co-contamination of the environment, presumably leading to a great health concern (Martinez, 2009).

Several proteins were found in multiple spots on the two gels. From the 42 different proteins identified in *van*A *E. durans* SG 2 isolate, five of them were involved in stress response. Usually, stress response requires heat molecular chaperones or shock proteins that preserve protein function or repair damage after cell injury. As such, the integrity of chaperone systems can seriously modify the progression of diseases associated with ageing, DNA damage and chronic injury (Mosser & Morimoto, 2004). While the molecular chaperone proteins are among the most evolutionarily preserved proteins and have a ubiquitous function in all repair processes, there is a high degree of tissue specificity in chaperone induction (Blake et al., 1991; Blake et al., 1990) showing that some cells have developed unique stress responses due to unique micro-environmental pressures. DnaK are abundant heat shock proteins that function as chaperones inside the bacterial cytoplasm (Gauthier et al., 2005). The bacteria that overproduce the dnaK protein at all temperatures undergo a considerably reduced heat-shock response at high temperature. The dnaK protein is identified as an inhibitor of the heat-shock response (Tilly et al., 1983) a very important reaction for the survival of bacteria such as enterococci, and that contributes to the antibiotic resistance. The protein dnaK was detected in spot 3 as linked to the *Enterococcus faecalis* strain (accession number Q835R7), the *Streptococcus agalactiae* serotype III strain (accesion number P0A3J2), the *Streptococcus thermophilus* strain (Q5M6D1), the *Streptococcus pyogenes* serotype M12 strain (Q1JKD6) and the *Streptococcus mutans* (O06942).

It is important to underline the presence of both dnaK and dnaJ proteins (spot 14 and spot 16) in *van*A *E. faecium* SG 50 strain as related to the *Yersinia pseudotuberculosis* serotype O:1b and to the *Enterococcus faecalis* (*Streptococcus faecalis*), respectively. DnaJ proteins participate actively in the response to hyperosmotic and heat shock by preventing the aggregation of the stress-denatured proteins and by disaggregating proteins, also in an autonomous, dnaK-independent fashion. DnaJ proteins are identified as co-chaperones because they help another family of chaperones (dnaKs) with protein folding. The dnaJ and dnaK proteins must act together to facilitate protein folding (Paulsen et al., 2003).

In vitro the GroEL proteins raise the yield of functional protein during refolding by suppressing aggregation as a side-reaction and, probably, by shifting the substrate protein from off-

pathway reactions back to the productive folding pathway (Sparrer et al., 1996). The GroEL protein is the major heat shock protein of a large number of bacteria and belongs to the chaperonin family. This protein avoids the misfolding of proteins and promotes the refolding and the proper assembly of unfolded polypeptiedes caused under stress condition (Paulsen et al., 2003). In the *van*A *E. faecium* SG 2 isolate, the spot 4 showed the presence of the groL protein (Q93EU6, Q8KJ20, Q8KJ18, Q8VT58 and Q8CX22) associated to the *Enterococcus faecalis*, the *Streptococcus anginosus*, the *Streptococcus constellatus*, the *Streptococcus gordonii*, the S*treptococcus agalactiae* serotype III, respectively. The dnaK and groL proteins were also detected in the enterococci strains from seagulls (Radhouani et al., 2010b) and in the *Salmonella* strains from wild boars (Pinto et al., 2010).

In the analysis of the obtained proteomes, the most abundant proteins identified in our *van*A SG 2 and *van*A SG 50 strains were those involved in glycolysis. In our study, the high number of proteins linked to the ATP synthesis, transferase, translation and protein folding is emphasized.

It is highly important to point out that proteins from *van*A enterococcal strains were identified in the obtained proteomes, some of which some are involved in the antibiotic resistance. These proteins were controled by vanomycin which, also, triggered innate signal regulators, adhesion factors, and metabolic gene expression in *E. faecalis*. Therefore, these responses may enable *Enterococcus* spp. to adapt, survive and remain pathogenic even under the pressure of the vancomycin treatment. Our results are in accordance with those observed in another report (Wang et al., 2010).

VI.2.6. Concluding remarks

Our report showed that it became possible through a detailed proteomic approach and a 2-DE combined with mass spectrometry (MALDI/TOF-TOF) to obtain important information for further understanding of antibiotic-resistant mechanism(s), but also for the evaluation of protein profiles in response to various stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance. All of these could represent a valid and integrating approach for the development of new therapeutic strategies (Hiscock & Upton, 2000). In fact, the elaboration of a 2-DE electrophoresis gel of the 2 *van*A enterococcal strains with phenotypic and genotypic profiles, indicating antimicrobial resistance, permitted us to identify and characterize the present proteins.

VI.2.7. Supplementary material

Supplementary material (Tables S7 and S8) regarding this manuscript is available at JIOMICS online (http://www.jiomics.com/index.php/jio/rt/suppFiles/86/0).

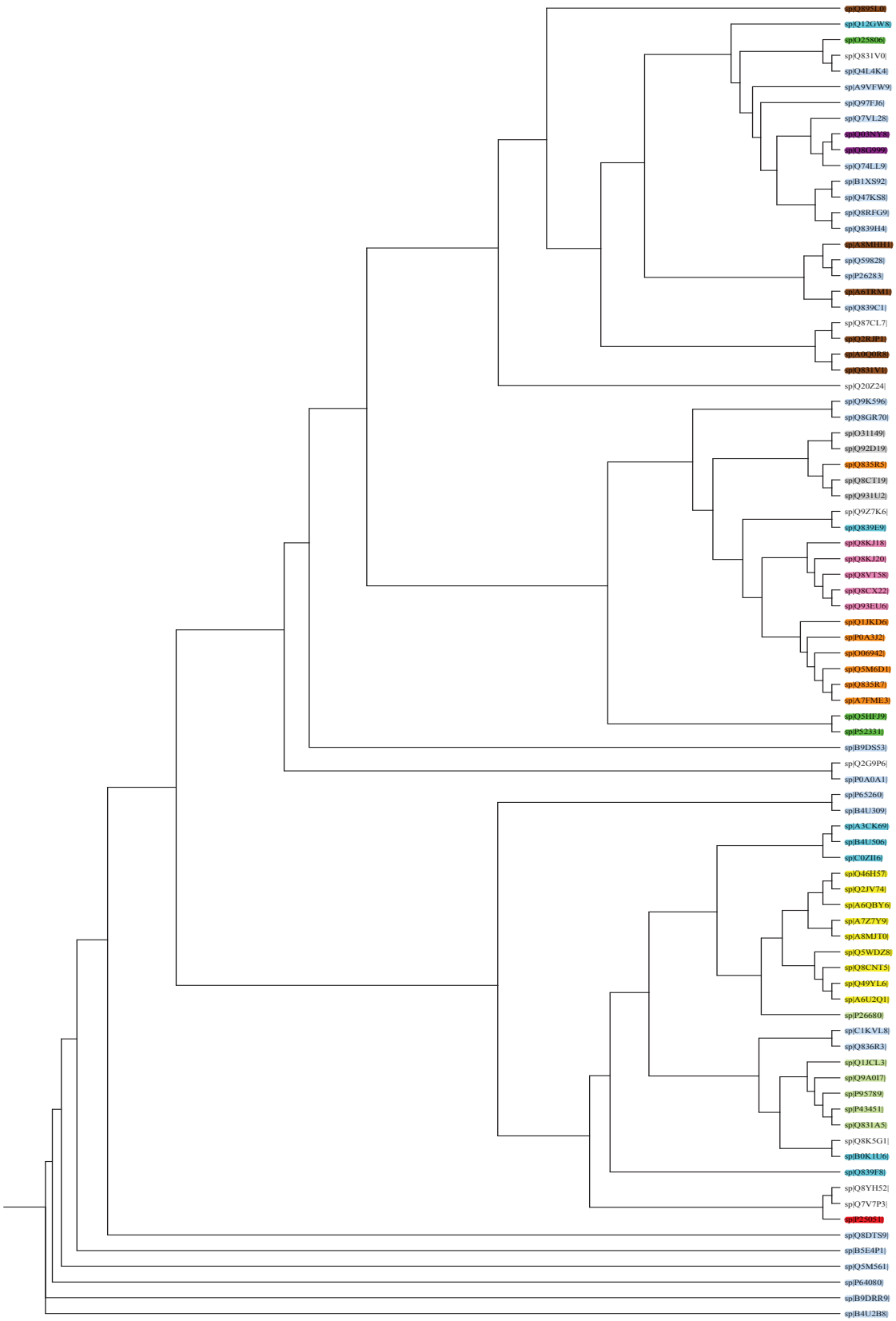

Figure VI.2.3 | Phylogenetic tree of FASTA protein sequences of all proteins identified
The full alignment of these sequences were done with MUSCLE (v3.7) configured for highest accuracy

VI.2.8. Acknowledgements

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VI.2.9. References

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PART IV

CONCLUSION

CHAPTER VII

GENERAL DISCUSSION & CONCLUSIONS

General discussion and conclusions

In the present thesis, genomic and proteomic tools were used to perform the molecular characterization of antimicrobial resistance mechanisms in *E. coli* and enterococci. The prevalence of antimicrobial resistance and the mechanisms implicated were studied in randomly-selected faecal *E. coli* and *Enterococcus* spp. isolates of wild animals in Portugal. The aim of this research was also to analyse the faecal carriage of ESBL-producing *E. coli* and VRE isolates recovered from these animals and to identify the type of ESBL encoding genes and *van* genes. Additionally, the identification of other associated resistance genes, *E. coli* phylogenetic groups, enterococcal species identity and the detection of virulence determinants were also focused in this study.

Since the 1950s, antimicrobial use in clinical and veterinary practice has been the major selective force for emergence and dissemination of resistant bacteria and resistance genes (Bonomo & Rossolini, 2008; Thaller et al., 2010). However, bacteria may acquire resistance determinants without direct exposure to an antimicrobial through horizontally mobile elements including conjugative plasmids, integrons and transposons (Middleton & Ambrose, 2005). The dynamics of spreading and the ecology of acquired antimicrobial resistance (mediated by resistance determinants that are not present usually in bacteria) are very complex (Singer et al., 2006) and difficult to elucidate. Some of the main parameters that regulate the opportunity for bacterial populations to develop and maintain antimicrobial resistance are: the horizontal transfer of antimicrobial resistance genes along with mutation; and the fitness effects of resistance and compensatory mutations, both in the presence and absence of antimicrobials (MacLean et al., 2010).

Recently, interesting insights into this field have been provided by studies on the presence of acquired resistance traits in the commensal microbiota of various wildlife animals (Pallecchi et al., 2008), frequently mammals and birds from either remote place (Thaller et al., 2010). Most antimicrobials used nowadays, and the respective resistance genes, have originated from the environment, particularly from the soil, and have existed for a long time in nature. For example, genes for penicillin resistance (β-lactamases) and vancomycin resistance are estimated to have been evolved for million of years (Waksman & Woodruff, 1940; Wright, 2010). Co-selection with other resistances, particularly to fluoroquinolones, aminoglycosides and sulfonamides, seem to have contributed to the increasing antimicrobial resistance problem. The occurrence of high-risk clones harbouring several β-lactamases simultaneously (ESBLs, metallo-beta-lactamases or cephamycinases) simultaneous to the emergence of new resistance mechanisms to fluoroquinolones and aminoglycosides, demands future surveillance studies (Coque et al., 2008a).

In the present research, the prevalence of ESBL-producing *E. coli* isolates in wild animals (seagulls, common buzzards and red foxes) was 12.7% (19.3%, 15.2% and 4%, respectively). Moreover, the occurrence of VRE strains in these wild animals was 20.4% (10.5%, 36.4% and 21.2%, respectively). Concerning ESBL-producing *E. coli*, a lower rate was observed in wild birds

from Germany (5.2%) and Mongolia (4.5%) (Guenther et al., 2012), but the same rate was observed in a previous study of the group in different wild animals from Portugal (12.5%) (Costa et al., 2006). VRE prevalence was also reported in glaucous gulls from Alaska (6%) (Drobni et al., 2009), in woodmice and badgers from England (4.6% and 1.2%, respectively) (Mallon et al., 2002). More recently, VRE prevalence was reported at Portugal in wild birds (3.2%) (Silva et al., 2011), in Iberian wolves and Iberian lynxes (2.2%) (Gonçalves et al., 2011) and in gilthead seabream (5.9%) (Barros et al., 2012).

Regarding *Enterobacteriaceae,* the most commonly encountered ESBLs belong to the TEM, SHV and CTX-M families (Paterson & Bonomo, 2005). The SHV and TEM ESBL emerged by mutations of the broad spectrum TEM-1 and SHV-1 genes that were then transferred between bacteria through plasmids, which were in turn spread by clonal distribution among hospitals and countries.

The $bla_{\text{TEM-52}}$ gene, encoding an ESBL, was first described in 1998 in France (Poyart et al., 1998). Since then, it has been detected in clinical isolates, in production animals (Bielak et al., 2011), as well as in wild animals (Costa et al., 2004; Garmyn et al., 2011; Literak et al., 2010; Sousa et al., 2011). Currently, regarded as the most important ESBL enzyme family are the CTX-M-type beta-lactamases, named after their capability to hydrolyze cefotaxime. They are supposed to originate from beta-lactamases from *Kluyvera* spp. and currently comprise more than 70 different CTX-M enzymes. In this work, the $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-32}}$ were the most identified β-lactamase genes among the ESBL-*E. coli* isolates, followed by the $bla_{\text{CTX-M-1}}$ (17.4%) and $bla_{\text{SHV-12}}$ (8.7%), $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-14a}}$ (4.3%) genes.

To date, nine different vancomycin resistance mechanisms have been described in enterococci: *van*A, B, C, D, E, G, L, M and N, whereas *van*C mechanism identified as specific of same enterococcal species (*E. gallinarum, E. casseliflavus/E. flavescens*). Among those, the most common types of acquired resistance are *van*A and *van*B, with *E. faecium* carrying the *van*A genotype as the most common combination (Nilsson, 2012). In fact, this was confirmed through the research performed within this thesis, where most of VRE isolates with acquired resistance corresponded to *van*A-*E. faecium* or *van*A-*E. durans*; the remaining VRE isolates recovered corresponded to the intrinsically resistant *E. gallinarum* species with the *van*C1 gene. It is important to point out that the presence of the *van*A determinant in enterococci is of relevance because this gene is generally located on mobile genetic elements that can be transferred to other microorganisms.

The wide range of identified ESBL and *van* genes in wild animals is still very restricted in comparison to clinical isolates of human and veterinary origin. Guenther et al. (Guenther et al., 2011) suggested that these results could reflect the small number of studies performed on wildlife to date, or that certain types of genes are more successful in the environment (due to co-selection with other non-resistance genes, for example). Another hypothesis that is supported by the similarity of

resistance profiles between wild animal and human/veterinarian clinical isolates, is that the ESBL and *van* genes found in wild animals simply reflect the ones that are most prevalent in human and veterinary clinics and in livestock farming. However, since the dissemination of ESBL and *van* genes is highly driven by horizontal gene transfer, the occurrence of identical ESBL and *van* genes could also be based on the spread of the respective plasmids that are randomly distributed throughout the environment (Guenther et al., 2011). Therefore, characterization of the clonal nature of strains isolated from different hosts, as well as an accurate identification and localization of the ESBL and *van* genes, need to be considered to unravel the basis of the ESBL-*E. coli* and VRE dissemination in wildlife.

The phylogenetic group analysis classified all *E. coli* isolates of this study into 4 groups: A (39.1%), B1 (27.3%), B2 (12.7%) and D (20.9%). Strains from groups B2 and D contained more virulence factors than strains from groups A and D (Johnson et al., 2001). Nowadays, these phylogenetic groups differ in their ecological niches, life-history (Gordon & Cowling, 2003) and in some other characteristics such as their ability to exploit different sugar sources, their antimicrobial-resistance profiles and growth rate (Carlos et al., 2010). A recent survey (Walk et al., 2007) demonstrated that the majority of *E. coli* strains that are able to persist in the environment belong to the B1 phylogenetic group (Carlos et al., 2010).

The MLST analysis in the *van*A- *E. faecium* isolates showed that almost all the VRE isolates were assigned to sequence types ST273 or ST262; included in CC17, a high-risk clonal complex that seems to be responsible for the worldwide emergence of nosocomial vancomycin-resistant *E. faecium* isolates. CC17 isolates are frequently associated to ampicillin and fluoroquinolone resistance, and in most cases carry the *esp* and *hyl* virulence factor genes; these characteristics could have favoured the global expansion of this clonal complex. The presence of *esp* and *hyl* genes was confirmed in this study; but other virulence factor genes were also identified such as *gel*E, *cpd* and *agg*. The fact that CC17 strains are well adapted to the hospital environment difficult its eradication, highlighting the importance of a rapid and effective control of these resistant-bacteria, in order to avoid the transmission of glycopeptide resistance to other highly relevant microorganisms (López et al., 2013). The presence of virulence factor genes in enterococci isolates that belonged to high-risk clonal complexes adds to the public health concerns that arise from the spread of VRE into wildlife.

Resistance to a variety of antimicrobials used in human and veterinary medicine, including resistance to streptomycin, ampicillin, tetracycline, sulfonamides, kanamycin, and gentamicin, has been detected in bacteria obtained from wildlife (Blanco et al., 2007; Cole et al., 2005; Kozak et al., 2009; O'Rourke, 2003). Several studies observed a similarity in the overall resistance profiles of wild animal isolates with human or veterinarian clinical isolates (Anderson et al., 2008; Costa et al., 2008; Literak et al., 2010; Sayah et al., 2005; Silva et al., 2010). In this research, the most prevalent non-ESBL and non-VRE resistance phenotypes in wildlife *E. coli* and enterococci are streptomycin-ampicillin-tetracycline and erythromycin-tetracycline, respectively. These patterns are very similar

to those identified in other wild animals such as wild rabbits (Silva et al., 2010) and wild birds (Guenther et al., 2010b; Radhouani et al., 2012), being also very common in human and livestock populations in Europe (Guenther et al., 2011).

The use of antimicrobials as growth promoters was banned in the EU since 2006; disease can frequently be prevented by using good husbandry rather than with the prophylactic antibiotic use. Tetracycline resistance was by far the most common type of resistance observed in *E. coli* and enterococci of the wild animal isolates of this study and this could be associated with farm origin. This is not unexpected since tetracycline is often used as a first-line antimicrobial in disease prevention in food animals, and its widespread use has likely contributed to high rates of resistance. While tetracycline resistance genes are on mobile genetic elements, they are transmissible between bacteria; it is expected that wild animals, such as red foxes, exposed to bacteria from poultry or other farm sources were colonized by antimicrobial-resistant bacteria or acquired tetracycline resistance traits through horizontal gene transfer. Furthermore, resistance to sulfonamides and streptomycin appears commonly in bacteria from animal farming (Kozak et al., 2009). Despite a lack of a major association with farm origin, it was nevertheless not surprising to detect resistance to these antimicrobials in red foxes confined to farms. Yet, the presence of resistance to some antimicrobials already banned, such as chloramphenicol, is explained by the fact that these resistance genes are known to persist in *E. coli* and enterococci isolates (Kozak et al., 2009).

Antimicrobial-resistant bacteria have been commonly isolated from a variety of sources, including domestic sewage, drinking water, rivers, and lakes (Sayah et al., 2005). Antimicrobials from both urban and agricultural sources persist in soil and aquatic environments, and the selective pressure imposed by these compounds may affect treatment of human diseases (Allen et al., 2010). In addition to the consequences for human health, interests and concerns have increased regarding contamination of surface water with resistant bacteria from livestock operations and human garbage.

Most of the previous antimicrobial resistance studies in wildlife have focused on resistance phenotypes and only recently genotyping has been performed in bacteria from wild animals. The presence of antimicrobial resistance genes such as *sul* (97.8% of SXT-resistant *E. coli*), *tet*(A) and/or *tet*(B) (81.7% of tetracycline-resistant *E. coli*), *aac*(3)-II and/or *aac*(3)-IV (76.9% in gentamicin-resistant *E. coli*), *cml*A (51.4% in chloramphenicol-resistant *E. coli*) and *aad*A (45.1% in streptomycin-resistant *E. coli*) has been reported in antimicrobial resistant ESBL- and non-ESBL-*E. coli* from the wild animals of this study. These resistance genes were also reported in other wild animals worldwide (Cole et al., 2005; Costa et al., 2008; Guenther et al., 2010a; Guenther et al., 2010b; Kozak et al., 2009; Silva et al., 2010). In this research, almost 75% of SXT-resistant *E. coli* isolates carried class 1 integrons, and 17% contained both class 1 and class 2 integrons. The high prevalence of integrons is a cause of concern, mainly due to the significant association of integrons with ampicillin resistance (even though ampicillin-resistant genes are not frequently included into integrons, as is the case of $bla_{\text{TEM-1}}$) and even with multi-resistance phenotypes. The presence of

integrons among commensal *E. coli* isolates from wild animals is of concern as this genetic structure is very efficient in the acquisition of antimicrobial resistance genes, which could be then transmitted to other bacteria by mobile elements such as plasmids and transposons.

Concerning antimicrobial resistance genes in the enterococci isolates (VRE and non-VRE) of this study, the *erm*(B), *tet*(M) and/or *tet*(L) were frequently identified together in the same strain. The *erm*(B) gene is usually linked with the *tet*(M) gene on the highly mobile conjugative transposon Tn*916*, which predominates in clinically important Gram-positive bacteria. Furthermore, macrolide and glycopeptide resistance genes have also been described on the same transferable genetic element in *E. faecium* isolates (De Leener et al., 2004). Since transposons can be transported through conjugation, transformation and transduction, these elements play a major role in antimicrobial resistance development as they often contain integron gene sequences, which are critical for the rapid dissemination of antimicrobial resistance genes among bacteria (Ochman & Jones, 2000). Other antimicrobial resistance genes were also identified in the enterococci isolates of wild animals: *ant*(6)-Ia (37.1% of streptomycin-resistant enterococci), *cat*A (27.6% of chloramphenicol-resistant enterococci), *vat*(D) and/or *vat*(E) (18.4% of QD-resistant enterococci). All high-level gentamicin- and kanamycin-resistant enterococci carried the *aac*(6´)Ie-*aph*(2´´)Ia and *aph*(3´)-IIIa, respectively. High-level gentamicin resistance eliminates the synergistic bactericidal effect with beta-lactams and causes a major reduction in efficient therapeutic options. Enzymatic modification of aminoglycosides is by far the main aminoglycoside resistance mechanism in clinical isolates of both Gram-negative and Gram-positive bacteria. The bi-functional aminoglycoside modifying enzyme (AME) AAC(6′)-Ie-APH(2″)-Ia found in enterococcal isolates implicate high-level resistance to virtually all clinically available aminoglycosides, except streptomycin (Rosvoll et al., 2012).

It is important to study antimicrobial resistance genotypes in order to identify the genes responsible for resistance in wild populations, to determine genetic associations, and maybe to relate the origin of genes to an external source, such as humans or animal species (Coque et al., 2008b).

From what we know so far there seems to be a great success of multiresistant bacteria in wildlife and the main question that needs to be examined is why this is the case. On one hand this might be explained by a high environmental pollution with antimicrobials and their residues, resulting in a low but constant antimicrobial pressure. Wildlife is usually not exposed to clinically used antimicrobial agents but they can acquire antimicrobial resistant bacteria through contact with humans, domesticated animals and the environment, where water polluted with faeces appears to be the most significant vector (Guenther et al., 2011). For this case, different forces exist that may contribute to this dissemination such as physical forces, human activities and wild animals. Physical forces like wind and watershed are considered significant drivers for antimicrobial resistance genes spreading - antimicrobials and their resistance genes have been extensively disseminated in the environment since before the introduction of antimicrobial chemotherapies, but human activities may have increased the prevalence of resistant bacteria in the air and water (Allen et al., 2010). Regarding

human activities, several reports have demonstrated that antimicrobial resistance rates are higher among animals living close to humans and agricultural areas than among wild animals living in more isolated regions (Allen et al., 2010; Blanco et al., 2007; Cole et al., 2005; Guenther et al., 2011; Guenther et al., 2010a; Kozak et al., 2009). Such contact is considered to be responsible for spreading of resistant bacteria and horizontal transfer of antimicrobial resistance genes among bacteria from wild animal populations. Bacterial isolates obtained from wildlife whose habitat is utilized by humans are more likely to be resistant to antimicrobials than isolates in more pristine areas farther from humans or agricultural infrastructure (Kozak et al., 2009). Moreover, wild animals provide a biological mechanism for the dissemination of antimicrobial resistance genes; proximity to human activities influences the antimicrobial resistance profiles of gut bacteria of wild animals, which live in closely populated microbial habitats. For instance, wild birds such as seagulls and common buzzards studied in this research, represent a reservoir of antimicrobial-resistant bacteria with the potential for long-distance dissemination. Furthermore, the presence of antimicrobial resistant bacteria in aquatic environments affected by human and animal wastewater and soil provides evidence for this hypothesis (Kummerer & Henninger, 2003). In this context the common use of antimicrobials in aquaculture of fish is also of utmost importance due to possible direct influences on water birds (Smith, 2008). Wild birds, and migratory waterfowl specially, can travel long distances and inhabit an extensive variety of environments, from agricultural lagoons to remote mountain lakes, and can possibly spread antimicrobial resistance genes along the way. Geese and gulls nesting near waste or agricultural water harbour more antimicrobial-resistant *E. coli* and enterococci than do birds associated with unpolluted water (Allen et al., 2010; Cole et al., 2005; Dolejska et al., 2007). Their proximity to human activity raises the number of the antimicrobial-resistant bacteria that are associated with wild birds. Moreover, antimicrobials are used in various settings for food production. In fact, animals are treated with antimicrobials for curing disease, aquaculture relies on antimicrobials to manage infectious disease (Cabello, 2006), and fruit trees are often treated prophylactically with antimicrobials to control bacterial infections (McManus et al., 2002). In each of these situations, the consequences of the antimicrobials extend beyond the site of use. Antimicrobials applied in animal farming operations leach into waterways and groundwater; in many aquaculture settings, antimicrobials diffuse into the water surrounding the pens; and antimicrobials sprayed on plants can drift aerially (Allen et al., 2010).

On the other hand, antimicrobial resistance might not be the only source for the successful spreading of antimicrobial-resistant bacteria. Recent research literature reviews a number of non-resistance-related factors, such as bacterial virulence, phylogenetic background and numerous metabolic features to be involved.

To fill the multiple gaps that remain in understanding bacterial resistance, proteomics tools have been used to study bacterial physiology in response to antimicrobial stress. The large-scale analysis of changes in the protein composition of bacterial cells in response to treatment with

antimicrobial agents has made it possible to construct a database of proteins implicated in the process of resistance to drugs with similar mechanisms of action.

To understand how resistance manifests itself at the molecular level, the whole-proteome analysis of an ESBL-producing *E. coli* strain stressed by cefotaxime was obtained through a proteomics approach. In this research the break point of cefotaxime corresponded to 2 µg/ml. This study indicates increased and decreased percentages of a wide range of proteins, with a diverse secondary response to treatment with cefotaxime. Because of the function of OmpX in membrane permeability, these results suggest that this protein could be involved in controlling antimicrobials penetration such as β-lactams and fluoroquinolones through the enterobacterial outer membrane (Dupont et al., 2007). The presence of FtsZ is of great interest as its level dictates the frequency of cell division (Bi & Lutkenhaus, 1991). Furthermore, FtsY, also identified in the stressed strain, is a signal recognition particle receptor in *E. coli* that mediates the targeting of integral membrane proteins to translocons, by interacting with both signal recognition particle (SRP)-nascent polypeptide–ribosome complexes and the cytoplasmic membrane (Maeda et al., 2008). The results of the current work showed that the stressed strain has acquired defense mechanisms normally related to stress response, and the increasing numbers of proteins related to antimicrobial resistance confirmed an increased tolerance to antimicrobials. In this research, a proteomic qualitative study was realized in ESBL-stressed strain; it will be interesting to study the subproteome of ESBL-stressed strain in order to report the overall changes in gene expression. It might be expected that this cefotaxime-resistant bacteria could show alterations in drug binding to their cellular targets; alterations in cell membrane permeation of Gram-negative bacteria or, more frequently, high-level expression of extended spectrum beta- lactamases (ESBLs). An important aim of proteome analysis is to study the level of the expressed genes and their response to environmental changes. Membrane protein researches are of primary importance to understand how nutrients are transported inside the cell, how toxic molecules such as antimicrobial agents are exported, and the mechanisms of photosynthesis and energy metabolism. Proteomics can complement genomics, by characterizing gene products and their response to a variety of biological and environmental influences (Nouwens et al., 2000).

The numerous changes in protein levels observed in the stressed strain suggest that resistant microorganisms may develop molecular changes in an effort towards adaptation to adverse environmental conditions (Justice et al., 2008; Linares-Rodriguez & Martinez-Menendez, 2005), with implications for several aspects of the bacterial metabolism. Antimicrobial resistance often confers a metabolic cost, as resistance mutations typically occur in genes of target molecules with essential functions in the cell.

In this study, the proteome analysis of a *van*A-containg *enterococcus* strains resulted in a high frequency of proteins associated with the processes of glycolysis, conjugation, translation, protein biosynthesis, oxidation-reduction processes, stress response, antimicrobial resistance, among

others. This demonstrated an alteration in cell physiology in all processes that fulfill an important function in maintaining normal cell function. It is interesting to point out that the vancomycin/teicoplanin A-type resistance protein VanA was identified in the whole-proteome analysis of enterococci strains. VanA is a protein capable of utilizing both hydroxyl acids and D-Ala as substrates with a concomitant switch from ester to peptide bond formation dependent on pH (Dutka-Malen et al., 1995). The proposed glycopeptide mechanism of action commonly implicates the inhibition of peptidoglycan synthesis, which binds at the D-Ala-D-Ala dipeptide terminus of the nascent peptidoglycan (Eirich et al., 2011). The binding of glycopeptides at this target blocks processing by penicillin-binding proteins (proteins involved in the final stage of peptidoglycan synthesis) and thus affects cell wall biosynthesis. This process causes changes in osmotic pressure and makes the bacterial cell susceptible to lyses. Other mechanisms of action have been detected for glycopeptides, suggesting that this antimicrobial class can interact with different bacterial cell targets (Kahne et al., 2005; Lima et al., 2013).

Furthermore, this research reported the presence of proteins with more specific functions like molecular chaperones (DnaK, WrbA, GroEL, etc.) were found, showing that these proteins related to stress response assist in the correct folding and assembly of proteins and are implicated in various cellular processes including DNA replication, RNA transcription, flagella synthesis and UV mutagenesis (Yura et al., 1993). These proteins also participate in the immune response to bacterial infections and the development of autoimmune diseases (Murray & Young, 1992). Different classes of chaperones from different bacteria can directly induce cytokine expression and secretion in macrophages (Retzlaff et al., 1994). Other important data suggested that β-lactamase interacts with GroEL and GroES (Bochkareva et al., 1988; Laminet et al., 1990).

Considering that antibiotic treatment is our primary, and in several cases only, method of treating infectious diseases, this study is crucial to our future ability to fight infection. Furthermore, this research represents a sentinel study monitoring the impact of multiresistant bacteria on wild animals and their potential impacts on clinically important bacteria. These wild animals may represent an important reservoir of antimicrobial resistant bacteria and resistance genes. The several results obtained are a reflex of this situation. The evolution of this type of resistance in wild animals, and broadly in natural ecosystems, is of great importance in human and veterinary medicine, thus representing an alarming problem to human and animal health by transmission of these strains to waterways and other environmental sources via their faecal deposits. Therefore, it is important to prevent the use of antibiotics in order to limit the appearance and dissemination of antimicrobial resistant bacteria in wild ecosystems.

The understanding of how glycopeptide and cefotaxime affect protein expression in resistant bacteria can be particularly valuable. Not only is this understanding vital to studies of resistance mechanisms or stress tolerance (Wang et al., 2010) but also in finding novel bacterial cell targets, which could lead to the development of rational variants with improved bactericidal activity (Eirich

et al., 2011; Lima et al., 2013). The capability to identify protein components that are associated to specific phenotypes is a key step to analyse any biological process. Both antimicrobial resistance and virulence are often related with the presence of pathogenicity islands (Groisman & Ochman, 1996), which can be transferred to other strains by horizontal gene transfer (Ochman et al., 2000). This main mechanism can lead to the acquisition of new traits, which might be crucial in promoting its fitness and survival when exposed to changing environments (Shames et al., 2009). The identification of highly-abundant protein components such as those related to stress response or antimicrobial resistance can provide relevant feedback associated to the molecular features of the isolate (Tomazella et al., 2012).

Genomics approaches have led to the identification of mobile genomic islands, pathogenicity determinants, and antimicrobial resistance factors unique to each strain. Large-scale technologies such as proteomics, combined with sophisticated computational algorithms and databases have already put in the hands of researchers a more clear understanding of this complex (Tomazella et al., 2012). Proteomics offer a valuable complement to comparative genomics approaches by providing large-scale protein profiling and the ability to visualize changes in protein expression patterns in living cells. 2-DE is a well-established technique for separation and quantification of a large set of proteins and has much to contribute to the experimental investigation of microbial organisms. Yet it remains to be seen precisely how proteomics, alongside other genomic strategies, will aid in understanding the antimicrobial resistance mechanism and in the identification of new drugs. As limitations of proteomics continue to diminish as technologies advance, creativity in approaches to investigate new drug targets will be critical in taking advantage of available resources (Reddy et al., 2009).

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CHAPTER VIII

FUTURE PROSPECTS

Future prospects

To continue profiting from the current medical standards, the search for novel antimicrobials needs to be intensified in creative ways and new active compounds are urgently needed for the development pipelines. Unfortunately, the rising resistance problem matches with decreasing numbers of new antimicrobials reaching market approval (Wenzel & Bandow, 2011). Considering a decade-long research and development process, different approaches need to be integrated to accelerate discovery and development. The genomics approach was promising but did little to populate the clinical development pipeline and still did not produce marketed antimicrobials. However, compounds stemming from genomics-based discovery programs can also benefit from targeted proteome analysis; proteomic signatures further assist the in vivo mechanism of action of these compounds using high-throughput in vitro assays or inhibitors from compound improvement programs (Wenzel & Bandow, 2011).

Usually, to each antimicrobial agent corresponds an individual protein expression profile that makes it possible to construct a database of proteins related to antimicrobial resistance process. Though, it should be noted that specific modifications might occur in bacteria in response to specific antimicrobials (Bandow et al., 2003; Wenzel & Bandow, 2011; Xiong et al., 2010). These alterations cannot be detected by proteomic approaches, due to technical limitations that may be resolved by using the novel sequencing performances applied to genomics and transcriptomics (Hornsey et al., 2011). This necessitates a serious re-evaluation of the current approaches toward antibacterial drug discovery and use.

In this respect, there is an urgent need to develop mutual interdisciplinary protocols to evaluate resistance, as well as searching for novel methodologies and techniques that could help in understanding the most exciting and challenging issues (Lima et al., 2013). Sensitivity ultrasequencing will have a multi-dimensional impact on several aspects of the antimicrobial development field including, but not limited, to new drug target identification, understanding the mechanism of antimicrobial action, drug safety and efficacy assessment, bacterial resistance development, understanding the pathogenesis process, optimizing antimicrobial biosynthetic process, and devising personalized treatments for specific instances of infectious disease (Amini & Tavazoie, 2011).

Gene expression researches have clearly been at the forefront and modern techniques have provided a high-resolution view of various aspects of transcriptome organization, being extremely pertinent to the identification and cross-analysis of data engendered by transcription and translation. Therefore, the identification of genes, mRNA, together with protein expression may help to get a better knowledge of the molecular mechanisms and signals implicated in bacterial resistance, leading to the development of designed novel drugs. Other questions may also involve metabolites that can be synthesized in response to low-concentrations of antimicrobials and must be evaluated by metabolomic technologies. This approach could also lead to a better understanding of resistance mechanisms (Lima et al., 2013).

Proteomic approaches are theoretically able to analyse an unlimited number of proteins in a single assessment; however there are still several challenges and it is imperative to create different optimization measures. These challenges include technical limitations, such as sensitivity and high throughput capacity of MS instruments to obtain a greater amount of information, as well as methodological limitations. In this field, other intrinsic factors must be taken into account, including the existence of several antimicrobial resistance mechanisms and also a high degree of cellular heterogeneity in each species (Lima et al., 2013). Although MALDI-TOF technique has occupied a central position in the methodologies developed for determination of secondary metabolites, proteins, and peptides, a complete interpretation is extremely difficult when performed in organisms in which the genome has not yet been mapped.

Liquid chromatography/mass spectrometry (LC/MS) has become a powerful technology in proteomics researches in drug discovery, including target protein characterization and discovery of biomarkers (Chen & Pramanik, 2009). This technique has numerous advantages over other proteomics methods, such as rapid performance, high sensitivity, and the possibility of analysing protein and peptide abundance levels, as well as discriminating ESBL strains (Sparbier et al., 2012). Though, a further validation of this MALDI-TOF MS-based resistance assay will be required because there are different bacterial species with different types of β-lactamases (Lima et al., 2013).

Wherever compounds with novel mechanisms of action will be discovered, proteomics will likely not be sufficient to identify the target or mechanism, but will contribute to elucidate the mechanism of action by generating hypotheses based on the stress response. Therefore, an interconnection of all "omics" may be indispensable to improve understanding and knowledge of antimicrobial resistance mechanisms. In short, it is important to point out that by using proteomics several significant results have been obtained, yet we remain a long way to understand all about bacterial resistance, and much progress can be expected in the following years when novel bacteria have their complete "omics" elucidated and compared.

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ATTACHMENTS

Table S9 | Protein spots identification of 2-DE gels and MALDI-TOF sequencing results from wild-type strain ESBL-*E. coli* C5478

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
1	DNA protection during starvation protein	*Escherichia coli* O139:H28	dps	A7ZJM7	18684	5.70	19	92	177	Stress response: Starvation	[1]
2	Ferric uptake regulation protein	*Escherichia coli* O157:H7	fur	P0A9B1	17012	5.68	5	41	61	Transcription	[2]
5	Universal stress protein G	*Escherichia coli* O157:H7	uspG	Q8XBT3	15926	5.89	7	66	125	Stress response: General stress	[2]
7	Peroxiredoxin osmC	*Escherichia coli*	osmC	P0C0L2	15193	5.57	4	31	65	Stress response: Oxidative stress	[3]
8	30S ribosomal protein S6	*Escherichia coli* O139:H28	rpsF	A7ZV71	15177	5.26	3	28	59	Translation	[1]
9	10 kDa chaperonin	*Escherichia coli* O139:H28	groS	A7ZV11	10381	5.15	6	74	69	Protein folding	[1]
12	UPF0313 protein ygiQ	*Escherichia coli* (strain K12)	ygiQ	Q46861	84223	9.24	7	13	100	Unknown function	[3]
14	Outer membrane protein X	*Escherichia coli* O157:H7	ompX	P0A919	67648	5.30	8	49	118	Stress response: Antibiotic resistance	[2]
17	L(+)-tartrate dehydratase subunit beta	*Escherichia coli* O157:H7	ttdB	Q8XBK5	22988	6.08	4	31	73	L(+)-tartrate dehydratase activity: Energy metabolism	[2]
18	Glyceraldehyde-3-phosphate dehydrogenase A	*Escherichia coli* O157:H7	gapA	P0A9B4	35681	6.58	17	51	154	Glycolysis	[2]
20	Thiol peroxidase	*Escherichia coli* O157:H7	tpx	P0A864	17995	4.75	7	58	96	Stress response: Oxidative stress	[2]
21	S-ribosylhomocysteine lyase	*Escherichia coli* (strain 55989 / EAEC)	luxS	B7LEA1	19576	4.98	7	44	63	Autoinducer synthesis	[1]
24	Inorganic pyrophosphatase	*Escherichia coli* O157:H7	ppa	P0A7B0	19805	5.03	4	22	77	Phosphate-containing compound metabolic process	[2]
25	Alkyl hydroperoxide reductase subunit C	*Escherichia coli* O157:H7	ahpC	P0AE10	20862	5.03	12	63	105	Stress response: Oxidative stress	[2]
26	Superoxide dismutase [Fe]	*Escherichia coli* O157:H7	sodB	P0AGD5	21310	5.58	8	55	104	Stress response: Oxidative stress	[2]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
28	Modulator of drug activity B	*Escherichia coli* O157:H7	mdaB	P0AEY7	21877	5.85	5	34	64	Stress response: Oxidative stress	[2]
30	Glutathione S-transferase	*Escherichia coli* O157:H7	gst	P0A9D3	22968	5.85	8	63	93	Transport	[2]
31	Oxygen-insensitive NAD(P)H nitroreductase	*Escherichia coli* (strain K12)	nfnB	P38489	23947	5.80	17	65	149	Stress response: Oxidative stress	[3]
32	F1845 adhesin operon regulatory protein	*Escherichia coli*	daaA	Q47133	9843	7.94	3	61	66	Transcription	[1]
33	Purine nucleoside phosphorylase deoD-type	*Escherichia coli* O139:H28	deoD	A7ZVS7	26161	5.39	11	68	79	Nucleoside metabolic process	[1]
34	F1845 adhesin operon regulatory protein	*Escherichia coli*	daaA	Q47133	9843	7.94	3	61	72	Transcription	[4]
35	Uncharacterized protein ycaC	*Escherichia coli*	ycaC	P21367	23200	5.20	60	30	5	Unknown function	[3, 5]
36	UPF0234 protein yajQ	*Escherichia coli* O127:H6	yajQ	B7UJP9	18301	5.94	5	37	103	Unknown function	[6]
39	Phosphoribosylaminoimidazole-succinocarboxamide synthase	*Escherichia coli* O139:H28	purC	A7ZPS1	27149	5.05	9	41	90	Purine biosynthesis	[1]
40	Lysine-arginine-ornithine-binding periplasmic protein	*Escherichia coli* (strain K12)	argT	P09551	28088	5.19	8	41	84	Transport	[1]
41	Cystine-binding periplasmic protein	*Escherichia coli* O6	fliY	P0AEN0	29021	5.29	9	31	84	Transport	[7]
42	Stringent starvation protein A	*Escherichia coli* O157:H7	sspA	P0ACA5	24346	5.22	12	47	111	Unknown function	[2]
43	Adenylate kinase	*Escherichia coli* O139:H28	adk	A7ZIN4	23628	5.55	8	40	67	Nucleotide biosynthesis	[1]
44	Triosephosphate isomerase	*Escherichia coli* O139:H28	tpiA	A7ZUD3	27126	5.64	9	50	75	Glycolysis	[1]
46	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*Escherichia coli* O127:H6	gpmA	B7ULM8	28539	5.85	13	48	123	Glycolysis	[6]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
48	Succinate dehydrogenase iron-sulfur subunit	*Escherichia coli* (strain K12)	sdhB	P07014	27379	6.31	7	30	72	Transport	[3]
49	Pyrimidine monooxygenase RutA	*Escherichia coli*	rutA	C9QZ64	40146	5.14	4	16	72	Pyrimidine biosynthesis	[8]
50	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*Escherichia coli* O127:H6	gpmA	B7ULM8	28539	5.85	7	33	116	Glycolysis	[6]
51	30S ribosomal protein S2	*Escherichia coli* O139:H28	rpsB	A7ZHQ9	26798	6.61	9	44	65	Translation	[1]
52	Aerobic respiration control protein ArcA	*Escherichia coli* O157:H7	arcA	P0A9Q3	27389	5.20	10	43	110	Transcription	[6]
56	UPF0234 protein yajQ	*Escherichia coli* O127:H6	yajQ	B7UJP9	18301	5.96	3	29	64	Unknown function	[6]
57	3'(2'),5'-bisphosphate nucleotidase CysQ	*Escherichia coli* O157:H7	cysQ	Q8XCG6	27314	5.59	4	33	75	Sulfur metabolism	[2]
61	3-mercaptopyruvate sulfurtransferase	*Escherichia coli* O157:H7	sseA	P58388	30864	4.56	12	53	148	Sulfurtransferase activity	[2]
62	D-alanine--D-alanine ligase B	*Escherichia coli* (strain K12)	ddlB	P07862	32933	4.75	5	27	88	Cell shape	[3]
65	D-galactose-binding periplasmic protein	*Escherichia coli* O6	mglB	P0AEE6	35690	5.25	4	18	72	Transport	[1]
66	Putative ribosome biogenesis GTPase RsgA	*Escherichia coli* O139:H28	rsgA	A7ZV32	39440	5.59	4	17	75	GTP binding	[1]
67	Transaldolase B	*Escherichia coli* O157:H7	talB	P0A871	35368	5.11	10	42	84	Pentose shunt	[2]
72	Putative quinone oxidoreductase YhdH	*Escherichia coli* (strain K12)	yhdH	P26646	34873	5.63	9	45	118	Unknown function	[3]
73	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	*Escherichia coli* O139:H28	dapD	A7ZHQ6	30044	5.55	12	54	119	Amino-acid biosynthesis	[2]
74	Malate dehydrogenase	*Escherichia coli* O139:H28	mdh	A7ZSD0	32488	5.61	14	58	133	Tricarboxylic acid cycle	[1]
76	Putative glucose-6-phosphate 1-epimerase	*Escherichia coli* (strain K12)	yeaD	P39173	32874	5.89	9	25	84	Carbohydrate metabolism	[3]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
79	Cysteine synthase A	*Escherichia coli* O157:H7	cysK	P0ABK6	34525	5.83	18	78	225	Amino-acid biosynthesis, Cysteine biosynthesis	[2]
80	Putative glucose-6-phosphate 1-epimerase	*Escherichia coli* (strain K12)	yeaD	P39173	32874	5.89	9	25	84	Carbohydrate metabolism	[3]
83	Curved DNA-binding protein	EscherichiacoliO139:H28	cbpA	A7ZKA5	34404	6.33	3	17	57	Transcription	[1]
84	Xaa-Pro dipeptidase	*Escherichia coli* O8	pepQ	B7M650	50322	5.54	4	14	71	Proteolysis	[9]
88	Multiphosphoryl transfer protein	*Escherichia coli* O157:H7	fruB	P69812	39624	4.77	7	35	128	Transport	[2]
89	Protein kleA	*Escherichia coli*	kleA	Q52278	8636	5.08	3	42	71	Unknown function	[10]
92	Putative outer membrane porin protein nmpC	*Escherichia coli* (strain K12)	nmpC	P21420	40277	4.55	7	31	70	Transport	[3]
93	Alcohol dehydrogenase, propanol-preferring	*Escherichia coli* (strain K12)	adhP	P39451	35870	5.94	10	47	112	Stress response: Oxidative stress	[3]
94	Ribosomal RNA small subunit methyltransferaseG	*Shewanella piezotolerans*	rsmG	B8CVV5	23626	5.39	5	28	85	rRNA processing	[11]
95	3-oxoacyl-[acyl-carrier-protein] synthase 2	*Escherichia coli* O157:H7	fabF	P0AAI7	43247	5.71	11	43	81	Fatty acid biosynthesis	[2]
97	Phosphoglycerate kinase	*Escherichia coli* O139:H28	pgk	A7ZR34	41264	5.08	18	57	137	Glycolysis	[1]
98	Maltose-binding periplasmic protein	*Escherichia coli* O157:H7	malE	P0AEY0	43360	5.22	13	45	108	Transport	[2]
102	Aminomethyl transferase	*Escherichia coli* O139:H28	gcvT	A7ZR14	40235	5.36	16	52	172	Glycine catabolic process	[1]
103	Aspartateamino transferase	*Escherichia coli*	aspC	P00509	43831	5.54	11	38	112	Aspartate biosynthetic process	[1]
105	ATP synthase subunit alpha	*Escherichia coli* O139:H28	atpA	A7ZTU6	55416	5.80	12	31	135	Transport	[1]
108	Serine hydroxymethyl-transferase	*Escherichia coli* O139:H28	glyA	A7ZPZ4	45459	6.03	10	26	84	Amino-acid biosynthesis	[2]
109	Der GTPase-activating protein YihI	*Escherichia coli* O7:K1	yihI	B7NV06	19034	6.37	4	28	62	Ribosome biogenesis	[9]
110	Elongation factor Tu 1	*Escherichia coli* O139:H28	tuf1	A7ZSL4	43457	5.30	17	54	164	Protein biosynthesis	[1]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
111	Isocitrate dehydrogenase [NADP]	*Escherichia coli* (strain K12)	icd	P08200	46070	5.15	20	47	138	Tricarboxylic acid cycle	[1]
111	30S ribosomal protein S20	*Clostridium difficile* (strain 630)	rpsT	Q182G1	9777	11.17	6	60	36	Translation	[12]
114	Glutamate decarboxylase alpha	*Escherichia coli* O6	gadA	P69909	53221	5.22	20	129	129	Stress response: acid resistance	[7]
114	Glutamate decarboxylase beta	*Escherichia coli* O157:H7	gadB	P69911	53204	5.29	20	41	127	Stress response: acid resistance	[2]
115	Glutamate-1-semialdehyde2,1-aminomutase	*Escherichia coli* O139:H28	hemL	A7ZHP6	45897	4.75	6	24	60	Porphyrin biosynthesis	[1]
117	Malate dehydrogenase	*Escherichia coli* O139:H28	mdh	A7ZSD0	32488	5.61	11	52	122	Tricarboxylic acid cycle	[1]
119	ATP synthase subunit beta	*Escherichia coli* O139:H28	atpD	A7ZTU4	50351	4.90	24	76	242	Transport	[1]
120	Uncharacterized protein ycfD	*Escherichia coli* (strain K12)	ycfD	P27431	42609	4.69	5	19	89	Unknown function	[3]
124	Uncharacterized zinc-type alcohol dehydrogenase-like proteinYahK	*Escherichia coli* (strainK12)	yahK	P75691	38524	5.80	4	12	73	Unknown function	[3]
126	Glutamate decarboxylase alpha	*Escherichia coli* O6	gadA	P69909	53221	5.22	12	34	91	Stress response: acid resistance	[7]
126	Glutamate decarboxylase beta	*Escherichia coli* O157:H7	gadB	P69911	53204	5.29	13	34	91	Stress response: acid resistance	[2]
127	ElongationfactorTu1	*Escherichia coli* O139:H28	tuf1	A7ZSL4	43457	5.30	9	33	100	Protein biosynthesis	[1]
128	Elongation factor Tu1	*Escherichia coli* O139:H28	tuf1	A7ZSL4	43457	5.30	9	33	100	Protein biosynthesis	[1]
131	Glyceraldehyde-3-phosphate dehydrogenase	*Salmonella typhi*	gapA	P0A1P1	35681	6.32	8	19	85	Glycolysis	[13]
132	Biotin carboxylase	*Escherichia coli* (strain K12)	accC	P24182	49745	6.65	10	29	87	Fatty acid biosynthesis	[3]
134	Dihydrolipoyl dehydrogenase	*Escherichia coli* O157:H7	lpdA	P0A9P2	50942	5.79	9	31	151	Glycolysis	[2]
135	Periplasmic dipeptide transport protein	*Escherichia coli* (strain K12)	dppA	P23847	60483	6.21	8	28	75	Transport	[3]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
137	Periplasmic oligopeptide-binding protein	*Escherichia coli* (strain K12)	oppA	P23843	60975	5.85	15	35	92	Transport	[3]
138	PyruvatekinaseI	*Escherichia coli* O157:H7	pykF	P0AD62	51039	5.77	18	48	160	Glycolysis	[1]
139	Succinate dehydrogenase flavoprotein subunit	*Escherichia coli* O157:H7	sdhA	P0AC43	65008	5.85	12	28	118	Transport	[2]
141	Fructose-bisphosphate aldolase class 2	*Escherichia coli* O157:H7	fbaA	P0AB72	39351	5.52	7	27	86	Glycolysis	[6]
142	Seryl-tRNA synthetase	*Escherichia coli* O139:H28	serS	A7ZJW2	48669	5.34	23	51	142	Protein biosynthesis	[1]
144	Phosphoenolpyruvate carboxykinase [ATP]	*Escherichia coli* O139:H28	pckA	A7ZST1	59863	5.46	9		108	Gluconeogenesis	[1]
145	Alkyl hydroperoxide reductase subunit F	*Escherichia coli* (strain K12)	ahpF	P35340	56484	5.47	12	32	96	Cell redox homeostasis	[3]
147	Oligopeptidase A	*Escherichia coli*	prlC	P27298	77461	5.15	8	14	110	Proteolysis	[3]
149	Phosphoenolpyruvate-protein phosphotransferase	*Escherichia coli* (strain K12)	ptsI	P08839	63750	4.78	22	43	166	Transport	[3]
150	60k Dachaperonin1	*Escherichia coli* O1:K1/APEC	groL1	A1AJ51	57464	4.85	13	40	125	Protein Refolding	[1]
151	Flagellin	*Escherichia coli* (strain K12)	fliC	P04949	51265	4.50	9	23	61	Innate immune response	[3]
152	Chromosome partition protein mukF	*Escherichia coli* O127:H6	mukF	B7UN08	50645	4.75	5	11	86	DNA replication and Cell division	[6]
153	Chaperone protein DnaK	*Escherichia coli* O139:H28	dnaK	A7ZHA4	69130	4.83	28	54	279	Stress response: General / Heat	[1]
154	30S ribosomal protein S1	*Escherichia coli* O157:H7	rpsA	P0AG69	61235	4.88	24	52	239	Translation	[2]
155	Glutaredoxin-2	*Escherichia coli* O157:H7	grxB	P0AC61	24449	7.72	4	41	85	Transport	[2]
157	Glycyl-tRNA synthetase beta subunit (GlyRS)	*Escherichia coli* O127:H6	glyS	B7ULB9	76936	5.29	26	42	223	Protein biosynthesis	[1]
159	Polyribonucleotide nucleotidyltransferase	*Escherichia coli* O139:H28	pnp	A7ZS61	77111	5.09	17	29	166	RNA processing	[1]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
160	Elongation factor G	*Escherichia coli* O139:H28	fusA	A7ZSL5	77704	5.24	21	40	175	Protein biosynthesis	[1]
161	Elongation factor G	*Shigella flexneri* serotype 5b	fusA	Q0SZX7	77672	5.24	27	51	180	Protein biosynthesis	[3]
162	Chaperone protein ClpB	*Escherichia coli* O157:H7	clpB	P63285	95697	5.37	30	42	194	Stress response: General / Heat	[2]
166	DNA-directed RNA polymerase subunit beta	*Escherichia coli* O139:H28	rpoB	A7ZUK1	150937	5.14	10	12	76	Transcription	[1]
167	Leucyl-tRNA synthetase	*Escherichia coli* O139:H28	leuS	A7ZJ31	97768	5.16	16	17	82	Protein biosynthesis	[1]
169	Formate acetyltransferase 1	*Escherichia coli* (strain K12)	pflB	P09373	85588	5.69	33	47	204	Carbohydrate metabolism	[3]
171	Threonyl-tRNA synthetase	*Escherichia coli* O139:H28	thrS	A7ZMI6	74680	5.80	17	28	100	Protein biosynthesis	[1]
172	Formate acetyltransferase 1	*Escherichia coli* (strain K12)	pflB	P09373	85588	5.69	23	34	181	Carbohydrate metabolism	[3]
173	Malate synthase G	*Escherichia coli* (strain K12)	glcB	P37330	80780	5.79	11	21	73	Tricarboxylic acid cycle	[3]
174	Catalase HPII	*Escherichia coli* (strain K12)	katE	P21179	84224	5.54	22	24	217	Hydrogen peroxide catabolic process	[3]
177	Aspartyl-tRNA synthetase	*Escherichia coli* O157:H7	aspS	Q8XCI7	66115	5.47	12	22	91	Protein biosynthesis	[2]
178	Transketolase 1	*Escherichia coli* (strain K12)	tktA	P27302	72451	5.43	9	14	81	Transketolase activity	[3]
183	Superoxide dismutase [Mn]	*Escherichia coli* O157:H7	sodA	P66828	23065	6.45	7	47	74	Superoxide metabolic process	[2]
185	Xaa-Pro dipeptidase	*Escherichia coli* O157:H7	pepQ	Q8X8I1	50335	5.60	12	33	75	Proteolysis	[2]

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Table S10 | Protein spots identification of 2-DE gels and MALDI-TOF sequencing results from stressed strain ESBL-*E. coli* C5478

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
190	Peroxiredoxin osmC	*Escherichia coli* (strain K12)	osmC	P0C0L2	15193	5.57	5	46	74	Stress response: Oxidative stress	[1]
191	Protein yhfA	*Escherichia coli* O157:H7	yhfA	P0ADX3	14678	5.52	3	44	68	Stress response: Oxidative stress	[2]
196	Outer membrane protein X	*Escherichia coli* O157:H7	ompX	P0A919	18648	5.30	6	43	76	Stress response: Antibiotic resistance	[2]
198	DNA protection during starvation protein	*Escherichia coli* O139:H28	dps	A7ZJM7	18684	5.70	13	66	164	Stress response: Starvation	[3]
200	DNA protection during starvation protein	*Escherichia coli* O139:H28	dps	A7ZJM7	18684	5.70	7	42	74	Stress response: Starvation	[3]
202	Protein translocase subunit SecD	*Escherichia coli* O157:H7	secD	P0AG91	66648	8.62	7	13	78	Transport	[2]
205	Thiol peroxidase	*Escherichia coli* O157:H7	tpx	P0A864	17995	4.75	8	64	87	Stress response: Oxidative stress	[2]
206	Glucose-specific phosphotransferase enzyme IIA component	*Escherichia coli* O6	crr	P69784	18240	4.73	6	52	100	Transport	[4]
207	Single-stranded DNA-binding protein (SSB)	*Escherichia coli* O157:H7	ssb	P0AGE2	18963	5.45	9	43	82	DNA damage	[2]
208	Single-stranded DNA-binding protein (SSB)	*Escherichia coli* O157:H7	ssb	P0AGE2	18963	5.45	5	27	97	DNA damage	[2]
209	Superoxide dismutase [Fe]	*Escherichia coli* O157:H7	sodB	P0AGD5	21310	5.58	8	55	99	Stress response: Oxidative stress	[2]
210	ATP-dependent Clp protease proteolytic subunit	*Escherichia coli* O139:H28	clpP	A7ZIJ5	23286	5.52	8	37	64	Stress response: General / Heat	[3]
211	Inorganic pyrophosphatase	*Escherichia coli* O157:H7	ppa	P0A7B0	19805	5.03	7	39	85	Phosphate-containing compound metabolic process	[2]
212	Alkyl hydroperoxide reductase subunit C	*Escherichia coli* O157:H7	ahpC	P0AE10	20862	5.03	10	53	85	Stress response: Oxidative stress	[2]
213	Transcriptional regulator slyA	*Escherichia coli* O127:H6	slyA	P0A4U5	16427	7.03	4	29	82	Transcription	[5]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
214	Uracil phosphorribosyl transferase	*Escherichia coli* O139:H28	upp	A7ZPU1	22576	5.32	7	34	61	Nucleoside metabolic process	[3]
214	Protein yhbO	*Escherichia coli* (strain K12)	yhbO	P45470	18904	5.27	6	51	65	Stress response: General	[1]
215	FKBP-type 22 kDa peptidyl-prolyl cis-trans isomerase	*Escherichia coli* (strain K12)	fklB	P0A9L3	22203	4.85	4	25	59	Protein folding	[1]
216	Uncharacterized protein ycaC	*Escherichia coli* (strain K12)	ycaC	P21367	23200	5.20	5	32	74	Unknown function	[1]
217	Purine nucleoside phosphorylase deoD-type	*Escherichia coli* O139:H28	deoD	A7ZVS7	26161	5.39	13	56	98	Nucleoside metabolic process	[3]
218	Flavoprotein wrbA	*Escherichia coli* O139:H28	wrbA	A7ZKA9	20832	5.59	8	61	103	Stress response	[3]
219	3'(2'),5'-bisphosphate nucleotidase CysQ	*Escherichia coli* O157:H7	cysQ	Q8XCG6	27314	5.59	7	48	86	Stress response: Oxidative stress	[2]
221	Stringent starvation protein A	*Escherichia coli* O157:H7	sspA	P0ACA5	24346	5.22	10	43	90	Unknown function	[2]
222	Lysine-arginine-ornithine-binding periplasmic protein	*Escherichia coli* (strain K12)	argT	P09551	28088	5.19	14	60	117	Transport	[1]
223	Glutaredoxin-2	*Escherichia coli* O157:H7	grxB	P0AC61	24449	7.72	4	26	74	Transport	[2]
225	Adenylatekinase	*Escherichia coli* O139:H28	adk	A7ZIN4	23628	5.55	9	49	91	Nucleotide biosynthesis	[3]
227	Uncharacterized protein ydhL	*Escherichia coli* (strain K12)	ydhL	P64474	9638	8.75	5	59	59	Unknown function	[1]
228	50S ribosomal protein L1	*Escherichia coli* O139:H28 (strain E24377A / ETEC)	rplA	A7ZUJ7	24714	9.64	10	47	58	Translation regulation	[3]
230	2,5-diketo-D-gluconic acid reductase A	*Escherichia coli* (strain K12)	dkgA	Q46857	31147	6.00	6	28	113	Ascorbate biosynthesis	[1]
231	Nitrate/nitrite response regulator protein narL	*Escherichia coli* O157:H7	narL	P0AF30	23912	5.73	7	48	75	Transcription	[2]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
232	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*Escherichia coli* O127:H6	gpmA	B7ULM8	28539	5.85	9	46	77	Glycolysis	[5]
234	Maltose-binding periplasmic protein	*Escherichia coli* O157:H7	malE	P0AEY0	43360	5.22	8	28	58	Transport	[2]
235	Pyridoxine 5'-phosphate synthase	*Escherichia coli* O157:H7	pdxJ	P0A795	26596	5.61	11	59	95	Pyridoxine biosynthesis	[2]
236	Transcriptional regulatory protein CpxR	*Escherichia coli* O157:H7	cpxR	P0AE89	26296	5.39	6	37	96	Transcription	[2]
237	Uncharacterized sugar isomerase yihS	*Escherichia coli* (strain K12)	yihS	P32140	47687	5.71	6	20	88	Mannose metabolic process	[1]
239	Ornithine carbamoyltransferase	*Escherichia coli* O6:K15:H31	argI	Q0T9E0	36736	6.62	5	19	81	Amino-acid biosynthesis	[6]
240	FKBP-type peptidyl-prolyl cis-trans isomerase slyD	*Escherichia coli* O157:H7	slyD	P0A9L1	21182	4.86	8	29	106	Protein folding	[2]
241	3-mercaptopyruvate sulfurtransferase	*Escherichia coli* O157:H7	sseA	P58388	30864	4.56	15	63	214	Sulfurtransferase activity	[2]
242	D-galactose-binding periplasmic protein	*Escherichia coli* O6	mglB	P0AEE6	35690	5.25	19	63	180	Transport	[4]
243	Elongation factor Ts	*Escherichia coli* O139:H28	tsf	A7ZHR0	30518	5.22	19	73	244	Protein biosynthesis	[3]
244	Transaldolase B	*Escherichia coli* O157:H7	talB	P0A871	35368	5.11	15	60	139	Pentose shunt	[2]
245	Enoyl-[acyl-carrier-protein] reductase [NADH] FabI	*Escherichia coli* O157:H7	fabI	P0AEK5	28074	5.58	16	58	127	Stress response: Antibiotic resistance	[2]
245	Chaperone protein hchA	*Escherichia coli* O81	hchA	B7MWF3	31271	5.63	11	38	93	Stress response: General / Heat	[7]
246	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	*Escherichia coli* O139:H28	dapD	A7ZHQ6	30044	5.55	18	59	204	Amino-acid biosynthesis	[3]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
247	Putative quinone oxidoreductase YhdH	*Escherichia coli* (strain K12)	yhdH	P26646	34873	5.63	13	45	100	Stress response: Oxidative stress	[1]
248	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	*Escherichia coli* O139:H28	accA	A7ZHS5	35333	5.76	11	46	97	Fatty acid biosynthesis	[3]
249	Malate dehydrogenase	*Escherichia coli* O139:H28	mdh	A7ZSD0	32488	5.61	7	30	82	Tricarboxylic acid cycle	[3]
250	6-phosphofructokinase isozyme 1	*Escherichia coli* O157:H7	pfkA	P0A797	35162	5.47	11	33	88	Glycolysis	[2]
251	Glyceraldehyde-3-phosphate dehydrogenase A	Escherichia fergusonii	gapA	B7LQ20	35689	6.61	10	34	71	Glycolysis	[7]
252	DNA-directed RNA polymerase subunit beta	*Escherichia coli* O139:H28	rpoB	A7ZUK1	150937	5.14	12	15	80	Transcription	[3]
253	Cysteine synthase A	*Escherichia coli* O157:H7	cysK	P0ABK6	34525	5.83	19	80	211	Amino-acid biosynthesis	[2]
253	Transaldolase A	*Escherichia coli* O157:H7	talA	P0A869	35865	5.89	10	31	65	Pentose shunt	[2]
254	Cysteine synthase A	*Escherichia coli* O157:H7	cysK	P0ABK6	34525	5.83	16	74	149	Amino-acid biosynthesis	[2]
254	Transaldolase A	*Escherichia coli* O157:H7	talA	P0A869	35865	5.89	19	73	179	Pentose shunt	[2]
256	ADP-L-glycero-D-manno-heptose-6-epimerase	*Escherichia coli* O139:H28	hldD	A7ZTH2	34985	4.80	16	61	178	Carbohydrate metabolism	[3]
257	Uncharacterized protein ybbN	*Escherichia coli* (strain K12)	ybbN	P77395	31885	4.50	10	32	79	Cell redox homeostasis	[1]
260	Multiphosphoryl transfer protein	*Escherichia coli* O157:H7	fruB	P69812	39624	4.77	7	35	59	Transport	[2]
261	Cell division protein ftsZ	*Escherichia coli* O157:H7	ftsZ	P0A9A8	40299	4.63	25	85	275	Cell division	[2]
262	Transcriptional regulator slyA	*Escherichia coli* O127:H6	slyA	P0A4U5	16427	7.03	4	29	82	Transcription	[5]
263	Putative outer membrane porin protein nmpC	*Escherichia coli* (strain K12)	nmpC	P21420	40277	4.55	10	35	105	Transport	[1]
266	Maltose-binding periplasmic protein	*Escherichia coli* O157:H7	malE	P0AEY0	43360	5.22	8	28	58	Transport	[2]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
267	NH(3)-dependent NAD(+) synthetase	*Escherichia coli*	nadE	B7L6L4	30791	5.30	12	38	106	NAD biosynthetic process	[7]
268	Phosphoglycerate kinase	*Escherichia coli* O139:H28	pgk	A7ZR34	41264	5.08	20	68	179	Glycolysis	[3]
269	DNA-directed RNA polymerase subunit alpha	*Escherichia coli* O139:H28	rpoA	A7ZSI4	36717	4.97	21	58	236	Transcription	[3]
270	Succinyl-CoA ligase [ADP-forming] subunit beta	*Escherichia coli* O139:H28	sucC	A7ZJA8	41652	5.37	23	57	227	Tricarboxylic acid cycle	[3]
271	Aminomethyl transferase	*Escherichia coli* O139:H28	gcvT	A7ZR14	40235	5.36	10	40	75	Glycine catabolic process	[3]
272	Aspartate aminotransferase	*Escherichia coli* (strain K12)	aspC	P00509	43831	5.54	15	41	162	Aspartate biosynthetic process	[1]
273	Elongation factor Tu 1	*Escherichia coli* O139:H28	tuf1	A7ZSL4	43427	5.30	24	68	246	Protein biosynthesis	[3]
274	Fructose-bisphosphate aldolase class 2	*Escherichia coli* O157:H7	fbaA	P0AB72	39351	5.52	9	32	57	Glycolysis	[2]
276	Fructose-bisphosphate aldolase class 1	*Escherichia coli* O6	fbaB	P0A992	38313	6.24	15	49	190	Glycolysis	[4]
277	Flagellar regulator flk	*Escherichia coli* (strain K12)	flk	P15286	36645	9.48	5	31	96	Regulation of gene expression	[1]
278	Isocitrate dehydrogenase [NADP]	*Escherichia coli* (strain K12)	icd	P08200	46070	5.15	14	35	94	Tricarboxylic acid cycle	[1]
279	Adenylosuccinate synthetase	*Escherichia coli* O139:H28	purA	A7ZV47	47543	5.32	22	56	226	Purine biosynthesis	[3]
280	6-phosphogluconate dehydrogenase, decarboxylating	*Escherichia coli* (strain K12)	gnd	P00350	51563	5.04	18	52	174	Pentose shunt	[1]
281	Thiol peroxidase	*Escherichia coli* O157:H7	tpx	P0A864	17995	4.75	6	45	71	Stress response: Oxidative stress	[2]
282	Glutamate decarboxylase beta	*Escherichia coli* O157:H7	gadB	P69911	53204	5.29	19	45	116	Stress response: acid resistance	[2]
283	ATP synthase subunit beta	*Escherichia coli* O139:H28	atpD	A7ZTU4	50351	4.90	21	66	253	Transport	[3]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
284	Trigger factor	*Escherichia coli* O139:H28	tig	A7ZIJ4	48149	4.82	27	59	287	Cell division	[3]
285	Ribose-phosphate pyrophosphokinase	*Escherichia coli* O157:H7	prs	P0A719	34425	5.23	14	47	143	Nucleotide biosynthesis	[2]
287	Protein klaB	*Escherichia coli*	klaB	Q52328	42130	6.05	7	19	114	Stress response: Tellurium resistance	[8]
288	60 kDa chaperonin 1	*Escherichia coli* O1:K1 / APEC	groL1	A1AJ51	57464	4.85	22	38	152	Protein refolding	[9]
289	Phosphoenolpyruvate-protein phosphotransferase	*Escherichia coli* (strain K12)	ptsI	P08839	63750	4.78	23	53	132	Transport	[1]
290	Flagellin	Shigella flexneri	fliC	Q08860	56603	4.51	20	46	182	Innate immune response	[10]
291	30S ribosomal protein S1	*Escherichia coli* O157:H7	rpsA	P0AG69	61235	4.88	31	60	271	Translation	[2]
292	Chaperone protein DnaK	*Escherichia coli* O139:H28	dnaK	A7ZHA4	69130	4.83	26	52	231	Stress response: General / Heat	[3]
293	Chaperone protein htpG	*Escherichia coli* O139:H28	htpG	A7ZIN3	71404	5.06	32	61	303	Stress response: General / Heat	[3]
294	Oligopeptidase A	*Escherichia coli* (strain K12)	prlC	P27298	77461	5.15	25	37	153	Proteolysis	[1]
295	Glutamine synthetase	*Escherichia coli* O157:H7	glnA	P0A9C7	52099	5.26	13	34	95	ATP-binding	[2]
296	Citrate synthase	*Escherichia coli* O6	gltA	P0ABH8	48383	6.21	10	26	79	Tricarboxylic acid cycle	[4]
296	Tryptophanase	*Escherichia coli* O139:H28	tnaA	A7ZTR3	53139	5.88	18	42	170	Tryptophan catabolism	[3]
297	Succinylornithine transaminase	*Escherichia coli*	astC	B7L6M2	44048	5.82	14	39	85	Arginine metabolism	[7]
298	Dihydrolipoyl dehydrogenase	*Escherichia coli* O157:H7	lpdA	P0A9P2	50942	5.79	19	39	158	Glycolysis	[2]
299	ATP synthase subunit alpha	*Escherichia coli* O139:H28	atpA	A7ZTU6	55416	5.80	23	53	215	Transport	[3]
300	Phosphoenolpyruvate carboxykinase [ATP]	*Escherichia coli* O139:H28 (strain E24377A / ETEC)	pckA	A7ZST1	59863	5.46	19	39	169	Gluconeogenesis	[3]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
301	Bifunctional purine biosynthesis protein PurH	*Escherichia coli* O8	purH	B7M7R5	57721	5.45	18	43	181	Purine biosynthesis	[7]
302	Elongation factor G (EF-G)	Shigella flexneri serotype 5b	fusA	Q0SZX7	77672	5.24	30	53	205	Protein biosynthesis	[11]
303	Polyribonucleotide nucleotidyltransferase	*Escherichia coli* O139:H28	pnp	A7ZS61	77111	5.08	19	30	116	RNA processing	[3]
304	Phosphoenolpyruvate synthase	*Escherichia coli* (strain K12)	ppsA	P23538	87836	4.93	28	37	234	ATP-binding	[1]
305	Leucyl-tRNA synthetase	*Escherichia coli*	leuS	B7L9I6	97800	5.11	16	20	108	Protein biosynthesis	[7]
306	Phenylalanyl-tRNA synthetase beta chain	*Escherichia coli* O157:H7	pheT	Q8XE32	88119	5.11	12	16	71	Protein biosynthesis	[2]
307	Aminopeptidase N	*Escherichia coli* (strain K12)	pepN	P04825	99313	5.14	12	16	70	Proteolysis	[1]
309	Pyruvate dehydrogenase E1 component	*Escherichia coli* O157:H7	aceE	P0AFG9	99948	5.46	24	25	190	Glycolysis	[2]
310	NADP-dependent malic enzyme	*Escherichia coli* (strain K12)	maeB	P76558	82878	5.34	23	41	183	Stress response: Oxidative stress	[1]
312	Chaperone protein ClpB	*Escherichia coli* O157:H7	clpB	P63285	95697	5.37	19	32	119	Stress response: General / Heat	[2]
312	Phosphate acetyltransferase	*Escherichia coli* (strain K12)	pta	P0A9M8	77466	5.28	26	45	204	Acetate biosynthetic process	[1]
313	Glycyl-tRNA synthetase beta subunit	*Escherichia coli* O127:H6	glyS	B7ULB9	76936	5.29	26	43	217	Protein biosynthesis	[5]
314	Cell division protein ftsY	*Escherichia coli* (strain K12)	ftsY	P10121	54480	4.46	16	42	162	Cell division	[2]
315	DNA-directed RNA polymerase subunit beta	*Escherichia coli* O139:H28	rpoB	A7ZUK1	150937	5.14	28	26	206	Transcription	[3]
316	Polyribonucleotide nucleotidyltransferase	*Escherichia coli* O139:H28	pnp	A7ZS61	77111	5.08	17	29	200	RNA processing	[3]
317	Carbamoyl-phosphate synthase large chain	*Escherichia coli* O157:H7	carB	P63737	118580	5.22	11	14	85	Amino-acid biosynthesis	[2]
318	DNA-directed RNA polymerase subunit beta	*Escherichia coli* O139:H28	rpoC	A7ZUK2	155918	6.67	46	35	265	Transcription	[3]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
319	Beta-galactosidase	*Escherichia coli* O139:H28	lacZ	A7ZI91	117300	5.20	12	16	65	Carbohydrate metabolic process	[3]
320	Isoleucyl-tRNA synthetase	*Escherichia coli* O139:H28	ileS	A7ZHB5	105013	5.66	35	38	225	Protein biosynthesis	[3]
321	Aldehyde-alcohol dehydrogenase	*Escherichia coli* O157:H7	adhE	P0A9Q8	96580	6.33	23	38	129	Alcohol metabolic process	[2]
322	Aconitate hydratase 1	*Escherichia coli* (strain K12)	acnA	P25516	98015	5.59	22	29	244	Tricarboxylic acid cycle	[1]
323	Aconitate hydratase 1	*Escherichia coli* (strain K12)	acnA	P25516	98015	5.59	15	26	95	Tricarboxylic acid cycle	[1]
323	Alanyl-tRNA synthetase	*Escherichia coli* O157:H7	alaS	Q8X3W8	96315	5.53	20	30	152	Protein biosynthesis	[2]
323	Putative monooxygenase ydhR	*Escherichia coli* (strain K12)	ydhR	P0ACX3	11281	5.09	3	23	28	Stress response: Oxidative stress	[1]
324	Catalase HPII	*Escherichia coli* (strain K12)	katE	P21179	84224	5.54	26	31	201	Hydrogen peroxide catabolic process	[1]
325	DNA gyrase subunit B	*Escherichia coli* O157:H7	gyrB	P0AES7	90179	5.72	18	28	96	Tricarboxylic acid cycle	[2]
325	Malate synthase G	*Escherichia coli* (strain K12)	glcB	P37330	80780	5.79	23	36	147	Stress response: Antibiotic resistance	[1]
326	Glycine dehydrogenase [decarboxylating]	*Shigella boydii* serotype 18	gcvP	B2U0S0	105070	5.62	26	30	170	Glycine metabolic process	[3]
327	Formate acetyltransferase 1	*Escherichia coli* (strain K12)	pflB	P09373	85588	5.69	37	51	291	Carbohydrate metabolism	[1]
328	Formate acetyltransferase 1	*Escherichia coli* (strain K12)	pflB	P09373	85588	5.69	30	41	249	Carbohydrate metabolism	[1]
329	Acetyl-coenzyme A synthetase	*Escherichia coli* (strain K12)	acs	P27550	72447	5.50	25	40	204	ATP-binding	[1]
329	Transketolase 1	*Escherichia coli* (strain K12)	tktA	P27302	72451	5.43	12	19	69	Transketolase activity	[1]
330	Transketolase 1 (TK 1)	*Escherichia coli* (strain K12)	tktA	P27302	72451	5.43	15	28	144	Transketolase activity	[1]
331	Chaperone protein ClpB	*Escherichia coli* O157:H7	clpB	P63285	95697	5.37	19	32	138	Stress response: General / Heat	[2]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
332	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	*Escherichia coli* O157:H7	glmS	Q8XEG2	67095	5.56	23	44	194	Carbohydrate biosynthetic process	[2]
335	Pyruvate kinase I	*Escherichia coli* O157:H7	pykF	P0AD62	51039	5.77	15	45	140	Glycolysis	[2]
336	CTP synthase	*Escherichia coli* O139:H28 (strain E24377A / ETEC)	pyrG	A7ZQM3	60792	5.63	11	24	103	Pyrimidine biosynthesis	[3]
337	Elongation factor Ts	*Escherichia coli* O139:H28	tsf	A7ZHR0	30518	5.22	9	33	77	Protein biosynthesis	[3]
338	Maltose-binding periplasmic protein	*Escherichia coli* O157:H7	malE	P0AEY0	43360	5.22	6	21	108	Transport	[2]
339	Phosphoglycerate kinase	*Escherichia coli* O139:H28	pgk	A7ZR34	41264	5.08	9	34	75	Glycolysis	[3]
340	Glutamate decarboxylase alpha	*Escherichia coli* O6	gadA	P69909	53221	5.22	6	18	78	Stress response: acid resistance	[4]
342	30S ribosomal protein S3	*Buchnera aphidicola* subsp. Acyrthosiphon kondoi	rpsC	P46172	22718	10.14	6	33	79	Translation	[12]
343	Phenylalanyl-tRNA synthetase alpha chain	*Acidovorax ebreus*	pheS	B9MHY2	39110	5.67	6	20	75	Protein biosynthesis	[13]
344	Phosphoglycerate kinase	*Escherichia coli* O139:H28	pgk	A7ZR34	41264	5.08	5	16	74	Glycolysis	[3]
345	6-phosphogluconate dehydrogenase, decarboxylating	*Escherichia coli* (strain K12)	gnd	P00350	51563	5.04	10	23	77	Pentose shunt	[1]
346	Arginyl-tRNA synthetase	*Escherichia coli* O157:H7	argS	Q8XCH2	64851	5.31	11	20	88	Protein biosynthesis	[2]
347	Malate synthase A	*Escherichia coli* (strain K12)	aceB	P08997	60521	5.39	12	30	97	Tricarboxylic acid cycle	[1]

Spot	Protein Description	Species	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count	Sequence coverage %	Protein Score	Biological function	References
348	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	*Escherichia coli* O157:H7	sucB	P0AFG7	43984	5.58	16	44	170	Tricarboxylic acid cycle	[2]
349	Phosphoglucomutase	*Escherichia coli* (strain K12)	pgm	P36938	58610	5.43	12	30	78	Carbohydrate metabolism	[1]
349	Seryl-tRNA synthetase	*Escherichia coli* O139:H28	serS	A7ZJW2	48669	5.34	19	47	145	Protein biosynthesis	[3]
350	Alkyl hydroperoxide reductase subunit F	*Escherichia coli* (strain K12)	ahpF	P35340	56484	5.47	13	32	131	Stress response: Oxidative stress	[1]
351	Glucose-6-phosphate 1-dehydrogenase	*Escherichia coli* O157:H7	zwf	Q8XCJ6	56040	5.55	12	28	90	Carbohydrate metabolism	[2]
352	N-succinylglutamate 5-semialdehyde dehydrogenase	*Escherichia coli*	astD	B7L6M0	53353	5.52	11	31	98	Arginine metabolism	[7]
353	Xaa-Pro dipeptidase	*Escherichia coli* O157:H7	pepQ	Q8X8I1	50335	5.60	7	24	54	Protein biosynthesis	[2]
353	Glutamyl-tRNA synthetase	*Escherichia coli* O139:H28	gltX	A7ZPK7	54181	5.59	13	33	110	Proteolysis	[3]
354	Histidyl-tRNA synthetase	*Escherichia coli* O139:H28	hisS	A7ZPV7	47285	5.65	14	40	153	Protein biosynthesis	[3]
355	Aspartyl-tRNA synthetase	*Escherichia coli* O157:H7	aspS	Q8XCI7	66115	5.47	7	16	81	Protein biosynthesis	[2]
356	Methionyl-tRNA synthetase	*Escherichia coli* O139:H28	metG	A7ZNT3	76648	5.56	9	21	76	Protein biosynthesis	[3]
357	Succinyl-CoA ligase[ADP-forming] subunit alpha	*Escherichia coli* O157:H7	sucD	P0AGF1	30044	6.31	8	48	104	Tricarboxylic acid cycle	[2]
358	Uncharacterized protein ygiC	*Escherichia coli* O157:H7	ygiC	P0ADT7	45282	4.67	11	36	119	Catalytic activity	[2]
359	Thioredoxin reductase	*Escherichia coli* O157:H7	trxB	P0A9P5	34829	5.30	10	47	72	Removal of superoxide radicals	[2]
360	Ribose-phosphate pyrophosphokinase	*Escherichia coli* O157:H7	prs	P0A719	34425	5.23	15	53	152	Nucleotide biosynthesis	[2]

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