Universidade de Aveiro Departamento de Biologia
2014

Diana Patrícia Pires Dias

Presence of pathogenic bacteria and antimicrobial resistance in Portuguese wild ungulates

Presença de bactérias patogénicas e resistência antimicrobiana em ungulados selvagens Portugueses

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Diana Dias

Universidade de Aveiro
2014

Departamento de Biologia

Diana Patrícia Pires Dias

Presence of pathogenic bacteria and antimicrobial resistance in Portuguese wild ungulates

Presença de bactérias patogénicas e resistência antimicrobiana em ungulados selvagens Portugueses

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e da Doutora Tânia Isabel Sousa Caetano, Bolseira de Pós-Doutoramento no Departamento de Biologia da Universidade de Aveiro.

Dedicada aos meus pais Deolinda e José, e ao meu irmão Rodrigo.

o júri

presidente

Prof. Doutora Maria Ângela Sousa Dias Alves Cunha
professora auxiliar, Universidade de Aveiro

Prof. Doutora Tânia Isabel Sousa Caetano
bolseira de Pós-Doutoramento, Universidade de Aveiro

Prof. Doutora Anabela de Oliveira Pereira
estagiária de Pós-Doutoramento, Cesam – Centro de Estudos do Ambiente e do Mar

agradecimentos

Em primeiro lugar, quero agradecer à Professora Sónia por aceitar ser minha orientadora, me integrar na família “LBM”, pela ajuda e orientação e por me permitir realizar este trabalho pelo qual tenho tanto gosto e interesse. Agradeço especialmente à Tânia por todos os ensinamentos, amizade e paciência. Por me ajudar sempre a encontrar uma solução para todos os meus “problemas”. Sei que não poderia ter tido melhores pessoas para me orientarem.

Agradeço ao Prof. Carlos Fonseca e à Rita Torres da Unidade de Vida Selvagem (UA) pela recolha do material em estudo, pela disponibilidade, ajuda e esclarecimentos.

Obrigada à Marta e à Cátia pelo apoio, amizade e diversão. À Joana B. e à Liliana por me ajudarem sempre que precisei. À Cláudia e ao Tiago “seu filho” pela amizade e pela animação no laboratório. Agradeço ainda ao restante grupo LBM (Teresa, Raquel, Andreia e Joana L.).

Agradeço ao Décio por todo o carinho, presença e amizade que foram fundamentais.

Um grande obrigada a todos aqueles a quem posso chamar de amigos, que partilharam comigo os últimos anos e me acompanharam não só nos bons momentos, mas também nos menos bons. Agradeço, à Rita, ao Fábio, ao Tito, ao 2º esquerdo (Ânia, Inês, Arminda, Rafa, Mila, Sá, Bitarães, Cardoso, Lili, Magalhães…), à Filipa, Telma, Vanessa, Susana, Eduardo, Diogo, Ana e à restante família Begeniense.

Quero por último agradecer aos meus pais, irmão, avós e padrinhos que me ajudaram e sempre demonstraram interesse na minha vida académica! ☺

palavras-chave

Resistência antimicrobiana, bactérias patogénicas, ungulados selvagens, amostras fecais, *E. coli*, *Salmonella*, STEC.

resumo

A resistência antimicrobiana é um problema emergente e global, tanto a nível clínico como veterinário. Em teoria, os animais selvagens raramente estão expostos a agentes antimicrobianos, e deste modo espera-se que a sua flora bacteriana apresente baixos níveis de resistência. Contudo, a crescente interação destes animais com atividades antropogénicas pode influenciar a aquisição de uma flora bacteriana resistente. *Escherichia coli* faz parte do trato intestinal de uma grande variedade de animais, incluindo o Homem. Esta bactéria pode disseminar-se facilmente em diferentes ecossistemas, sendo também um importante indicador da pressão seletiva exercida pela utilização de antimicrobianos. *Salmonella* spp. é uma bactéria patogénica, normalmente encontrada no intestino de diversos animais. Anualmente, na União Europeia são reportados à EFSA mais de 90,000 casos de salmoneloses.

O presente estudo foi realizado em três espécies de ungulados selvagens que habitam três localizações geográficas distintas em Portugal (Montesinho, Idanha-a-Nova e Lousã) e teve como objetivos: i) avaliar os níveis de resistência de isolados de *E. coli* ii) determinar o nível de ocorrência de *Salmonella* spp. e iii) determinar o nível de ocorrência de *E. coli* produtora da toxina shiga (STEC). Para tal foram recolhidas 67 amostras fecais de veado (n=41), javali (n=21) e corço (n=4). Numa primeira fase os isolados recolhidos foram tipados por BOX-PCR para selecionar estirpes geneticamente diferentes em cada amostra (n=152). Posteriormente realizou-se o teste de suscetibilidade a antimicrobianos (de acordo com o EUCAST). A deteção de *Salmonella* foi realizada de acordo com a norma ISO 6579:2002 Anexo D.

Os resultados obtidos revelaram que para *E. coli* se verificou resistência aos antibióticos ampicilina (10%), tetraciclina (9%), streptomicina (5%), cotrimoxazol (4%), amoxicilina/ácido clavulânico (2%) e cofoxitina (1%). Um fenótipo de multirresistência foi encontrado em 3.3% dos isolados, todos provenientes da região da Lousã. Os resultados foram também analisados de acordo com os valores de ECOFFs, tendo sido encontrados fenótipos do tipo não-selvagem para a ampicilina (10%), ceftazidima (6%), cotrimoxazol (4%), amoxicilina/ácido clavulânico (2%), aztreonam (1%) e cefoxitina (1%). No que se refere à pesquisa de *Salmonella*, os resultados revelaram uma baixa incidência na população estudada (1.5%). Esta estirpe revelou-se suscetível a todos os antimicrobianos testados.

Relativamente à presença de STEC, foi possível determinar que veados e corços dos três locais estudados são portadores deste tipo de estirpes. Detetaram-se três variantes do gene *stx* nos isolados STEC, incluindo *stx1c*, *stx2d* e *stx2g*. Foi ainda identificado o gene *ehxA,* que codifica para uma hemolisina, num isolado contendo a variante *stx2g.*

No seu conjunto, os resultados obtidos mostram que as populações de ungulados selvagens estudados são reservatórios de bactérias resistentes, assim como de bactérias potencialmente patogénicas e podem, por isso, atuar como veículo de transmissão entre a vida selvagem, o gado e o Homem.

keywords

Antimicrobial resistance, pathogenic bacteria, wild ungulates, faecal samples, *E. coli*, *Salmonella*, STEC.

Abstract

Antimicrobial resistance is as an emerging global problem in both human and veterinary medicine. In theory, wild animals are rarely exposed to antimicrobial agents and therefore low levels of AMR are to be expected. However, the growing interaction of these animals with anthropogenic activities can have a huge impact in their bacterial flora.

Escherichia coli is commonly found in the intestinal tract of a wide variety of animals and humans. This intestinal bacterium can be easily disseminated in different ecosystems. Therefore, it can be an useful indicator of the selective pressure exerted by the use of antimicrobials.

Salmonella is a pathogenic bacterium, commonly found in the intestine of healthy birds and mammals that can cause salmonellosis in humans. In the European Union, over 90,000 salmonellosis cases are reported every year to EFSA.

This study was conducted in wild ungulates from three distinct geographical areas in Portugal (Montesinho, Idanha-a-Nova and Lousã) and aimed to: i) access the levels of antibacterial resistance occurring in *E. coli* strains ii) determine the occurrence levels of *Salmonella* spp. and iii) determine the occurrence levels of shiga toxin-producing *E. coli* (STEC). To that purpose, a total of 67 faecal samples from red deer (n=41), wild boar (n=21) and roe deer (n=4) were collected. Before antibacterial susceptibility testing (according to the EUCAST guidelines), the *E. coli* isolates obtained were typed by BOX-PCR to select for genetically different strains for each sample (n=152). The detection of *Salmonella* was performed according to ISO 6579:2002 Annex D.

Results revealed that in *E. coli* resistance was observed to ampicillin (10%), tetracycline (9%), streptomycin (5%), co-trimoxazole (4%), amoxicillin/clavulanic acid (2%) and cefoxitin (1%). A total of 3.3% of the isolates exhibited a multiresistant phenotype, all from Lousã. The results were also analyzed according to ECOFFs. Non-wildtype phenotypes were obtained to ampicillin (10%), ceftazidime (6%), co-trimoxazole (4%), amoxicillin/clavulanic acid (2%), aztreonam (1%) and cefotxitin (1%). A low incidence of *Salmonella* spp. (1.5%) was observed and it was only identified in wild boar from Lousã. The isolate was susceptible to all the tested antimicrobials. Regarding the presence of STEC, it was possible to establish that red and roe deer from the three sampling sites carry this bacterium. The *stx* variants detected in the STEC isolates included *stx1c*, *stx2d* and *stx2g*. Moreover, the hemolysin gene *ehxA* was identified in a strain possessing the *stx2g* variant.

Overall, our results reveal that these populations of wild ungulates are reservoirs of antibiotic resistant and potential pathogenic bacteria. Therefore, these animals can act as dissemination vehicles between wildlife-livestock-human interfaces.

Table of contents

List of Figures iii
List of Tables v
List of Abbreviations vi
List of works presented/submitted as part of this dissertation viii
1. Introduction 1
1.1. Antibacterial agents 4
1.1.1. Inhibitors of cell wall synthesis 5
1.1.2. Inhibitors of protein synthesis 5
1.1.3. Anti-metabolites 6
1.1.4. Inhibitors of nucleic acid synthesis 7
1.2. General mechanisms of antibiotic resistance 7
1.3. Movement of antibiotic resistance in the environment 8
1.4. Antimicrobial resistance in wildlife 9
1.5. Etiologic agents 10
1.5.1. *Escherichia coli* 10
1.5.2. *Salmonella* spp. 15
1.6. Ungulates in study 16
1.6.1. Wild boar 16
1.6.2. Red deer 16
1.6.3. Roe deer 17
1.7. Sampling locations 17
1.7.1. Montesinho Natural Park 17
1.7.2. Lousã Region 17
1.7.3. Idanha-a-Nova 18
1.8. Objectives 18
2. Material and Methods 19
2.1. Flowchart and methodologies followed 21
2.2. Origin and description of the samples 21

2.3. Isolation and selection of *E. coli* isolates 22
2.3.1. Amplification of gadA/B (GAD) and uidA (GUD) genes 22
2.3.2. BOX-PCR fingerprinting 23
2.3.3. Conservation of bacterial isolates 24
2.4. Detection of *Salmonella* spp. 24
2.4.1. Amplification of *hilA* gene 25
2.5. Screening and selection of STEC isolates 26
2.5.1. DNA template preparation for PCR 26
2.5.2. Screening of STEC in faecal samples by PCR 26
2.5.3. Amplification of *rfbE*, *stx1, stx2, ehx* and *eae* genes in *E. coli* isolates 27
2.6. Antibiotic susceptibility testing 28
2.6.1. Preparation of inoculum 28
2.6.2. Preparation of plates and application of disks 28
3. Results and Discussion 31
3.1. Isolation and selection of *E. coli* isolates 33
3.2. Presence of *Salmonella* spp. 34
3.3. Antimicrobial susceptibility testing of *E. coli* isolates 35
3.3.1. Clinical Breakpoints 35
3.3.2. Epidemiological cut-offs (ECOFFs) 39
3.3.3. Resistance phenotypes 43
3.4. Screening of shiga toxin-producing *Escherichia coli* (STEC) 45
3.4.1. Samples 45
3.4.2. *E. coli* isolates 46
4. Conclusions and Future Perspectives 49
Bibliography 55
Appendices 65
Appendix 1. Typical composition of medium (g/litre) 67
Appendix 2. Samples data 69

List of Figures

Figure 1: Development of antibacterial resistance according to (CDC, 2013). 3
Figure 2: Representation of the site of action, targets of different classes of antibiotics (Lewis, 2013). 5
Figure 3: Schematic representation of the principal mechanisms of resistance to antibiotics (Allen *et al.*, 2010). 8
Figure 4: Schematic representation of factors involved in the dissemination of antibiotic resistance in the environment (Davies & Davies, 2010). 9
Figure 5: Percentage of *E. coli* isolates resistant to third-generation cephalosporins, by country in 2013 (European Centre for Disease Prevention and Control., 2014). 12
Figure 6: Mechanism of action of Shiga Toxin (Pacheco & Sperandio, 2012). 13
Figure 7: Model of the STEC transmission pathways from cattle to humans (Gansheroff & O'Brien, 2000)...14
Figure 8: Schematic representation of the experimental strategy used in this work. 21
Figure 9: Map of Portugal with the collecting sites indicated. 22
Figure 10: Percentage of the faecal samples from which *E. coli* were isolated. 33
Figure 11: Distribution of the positive faecal samples according to the animal species of origin..33
Figure 12: Example of a BOX-PCR fingerprint obtained in this study. The first and last lanes contain the DNA marker GeneRuler 1000bpplus (Thermo)...................................34
Figure 13: Percentage of resistant and intermediary *E. coli* isolates identified in this study according to clinical breakpoints (considering the total number of isolates). AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; S = Streptomycin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; TE = tetracycline; C = chloramphenicol..36
Figure 14: Percentage of resistant *E. coli* isolates from wild boar and red deer independently (roe deer was not included because no resistant isolates were identified). AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; S = Streptomycin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; TE = tetracycline; C = chloramphenicol. 38
Figure 15: Percentage of resistant *E. coli* isolates based on their geographical origin. AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; S = Streptomycin; NA =

nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; TE = tetracycline; C = chloramphenicol. 39
Figure 16: Percentage of non-wild type *E. coli* isolates according to epidemiological cut-offs, considering the total number of isolates. AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; C = chloramphenicol. 40
Figure 17: Percentage of non-wild type *E. coli* isolates according to epidemiological cut-offs, considering the isolates collected from wild boar, red deer and roe deer independently. AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; C = chloramphenicol. 41
Figure 18: Histograms of distribution of the inhibition zone diameters obtained for the *E. coli* isolates object of the present study and those provided by EUCAST to ampicillin, co-trimoxazole, cefotaxime and ceftazidime. The dashed line indicates the epidemiological cut-off value defined for the wild type population by EUCAST. 42
Figure 19: Histogram of the IZDs obtained for the *E. coli* isolates object of the present study to a) streptomycin and b) tetracycline. The dashed line indicates our proposal to ECOFF for the wild type population. 43
Figure 20: Percentage of *stx* variants detected in the 13 positive faecal samples. 46

List of Tables

Table 1: List of primers used in the PCR reactions for amplification of *gadA/B* and *uidA* genes....23
Table 2: List of primers used in the PCR reactions for amplification of *hylA* gene. 25
Table 3: List of primers used for STEC screening by Real-Time PCR....27
Table 4: List of primers used for the identification of STEC and for other pathogenicity genes in *E. coli* isolates. 28
Table 5: List of antibiotics used, respective concentration, respective ECOFFs and clinical breakpoints according to EUCAST or CLSI....29
Table 6: Resistance phenotypes detected among the 152 *E. coli* isolates obtained from faecal samples of wild ungulates in Portugal, according to clinical breakpoints....44
Table 7: Resistance phenotypes detected among the 152 *E. coli* isolates obtained from faecal samples of wild ungulates in Portugal, according to epidemiological cut-offs....44
Table 8: List of positive faecal samples for the shiga-toxin genes amplified in this study by real-time PCR. 45
Table 9: List of *E. coli* isolates positive for *stx* genes....47
Table 10: List of samples with respective collection date and location. 69
Table 11: List of samples with respective collection date and location. 70
Table 12: List of samples with respective collection date and location. 71

List of Abbreviations

AK	Amikacin
AMC	Amoxicillin/clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
ATM	Aztreonam
GUD	β-D-Glucuronidase
BPW	Buffered Peptone Water
CPE	Carbapenemase-producing *Enterobacteriaceae*
CO_2	Carbon dioxide
CTX	Cefotaxime
FOX	Cefoxitin
CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
C	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
SXT	Co-trimoxazole
DNA	Deoxyribonucleic acid
DHFR	Dihydrofolate reductase
ECOFFs	Epidemiological cut-off values
E. coli	*Escherichia coli*
EARS-Net	European Antimicrobial Resistance Surveillance Network
EARSS	European Antimicrobial Resistance Surveillance System
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EMA	European Medicines Agency
ESBLs	Extended spectrum beta-lactamases
EQA	External Quality Assessment
ExPEC	Extraintestinal pathogenic *E. coli*
GABA	gamma-Aminobutyric acid
Gb3	Globotriaosylceramide
GAD	Glutamate decarboxylase
GC	Guanine-cytosine

HUS	Hemolytic-uremic syndrome
HC	Hemorrhagic colitis
IPM	Imipenem
IZDs	Inhibition zone diameters
I	Intermediate resistance
ISO	International Organization for Standardization
LB	Luria-Bertani
MIC	Minimum inhibitory concentration
MSRV	Modified semisolid Rappaport-Vassiliadis
MHA	Mueller Hinton Agar
NA	Nalidixic acid
ON	Over-night
PCR	Polymerase chain reaction
RT-PCR	Real-time PCR
R	Resistant
SPI1	*Salmonella* Pathogenicity Island 1
SS agar	*Salmonella Shigella* agar
S	Sensitive
STEC	Shiga toxin–producing *E. coli*
NaCl	Sodium chloride
S	Streptomycin
TE	Tetracycline
UTIs	Urinary tract infections
UPEC	Uropathogenic *E. coli*
UVS	Wildlife Research Unit
XLD agar	Xylose Lysine Desoxycholate agar

List of works presented/submitted as part of this dissertation

Poster presentation:

Dias D., Torres R., Fonseca C., Mendo S., Caetano T. (2014) Assessment of antibacterial resistance levels and presence of pathogenic bacteria in Portuguese wild ungulates. Trends in Environmental Microbiology and Public Health 2014. 18-21th September. Lisbon (Portugal).

Oral presentation:

Torres R., **Dias D.**, Caetano T., Mendo S., Fonseca C. (2014) Antimicrobial resistance in wild boar (Sus scrofa) in Portugal. 10th International Symposium on Wild Boar and Other Suids. 1-5th September. Velenje (Slovenia)

ISI papers:

Dias D., Torres R., Fonseca C., Mendo S., Caetano T. (submitted) Assessment of antibacterial resistance levels and presence of pathogenic bacteria in Portuguese wild ungulates. Research in Microbiology.

1. Introduction

Antimicrobial resistance (AMR) is as an emerging global problem in both human and veterinary medicine. Its emergence is mainly due to massive antimicrobial use, selection and spread of resistant bacteria to the antimicrobial agents, either due to directly use in humans, as well as the use in food-production animals (Stefani, 2012). The origin of microbial resistance remains controversial: is it the result of human activity or rather a consequence of the antibiotic biosynthetic pathways that evolved over millions of years? This is a complex and challenging subject, existing evidences for both (Stefani, 2012). Some researchers believe that antibiotics have been produced for over 500 million years, dating back to the Cambrian period. Antibiotic-like molecules, are likely to be even older; the non-protein amino acids that are found as components of peptide antibiotics have been detected in meteorites and other primordial sources (Allen *et al.*, 2010). In the last few years, the intestinal flora of humans and animals have been recognized as an important reservoir of resistance genes (Figure 1), which are constantly subjected to different types, concentrations, and frequencies of antimicrobial agents (Sayah *et al.*, 2005). AMR has an adverse impact in clinical evolution, leading to higher costs due to the consumption of health related resources. More importance should be given to strengthening of hygiene and infection prevention (World Health Organization, 2014).

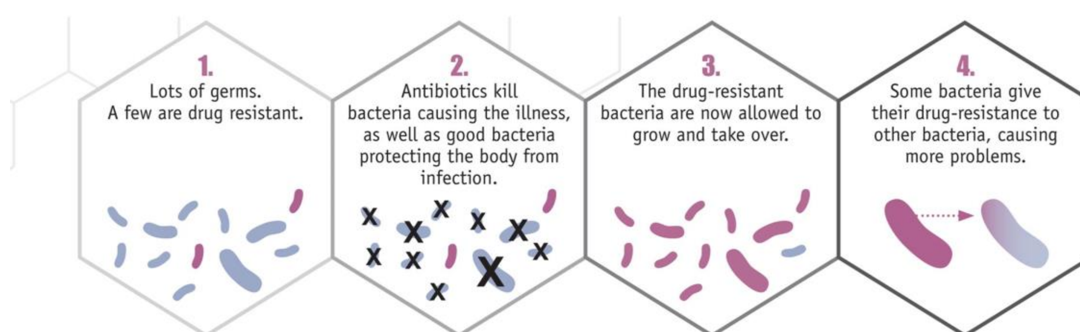

Figure 1: Development of antibacterial resistance according to (CDC, 2013).

The antibiotic classes used in animals for food production (as livestock, poultry and fish farming, sometimes with mass administration) are mostly the same as those that are used by humans (e.g. amoxicillin and erythromycin) (Allen *et al.*, 2010). The magnitude of the transmission of animal reservoirs for humans remains unknown and probably variable between different bacterial species (World Health Organization, 2014).

Effective monitoring is crucial to the efforts of national and international control of AMR. Tracing the use of antibiotics and the emergence of the spread of resistant strains

are important tools needed to guide intervention policies and evaluate the actions taken to promote appropriate antimicrobial use at all levels, locally and globally. However, there is a long way to go before antimicrobial use and antimicrobial resistance surveillance are established worldwide. In countries with limited health resources and comparatively weak systems, there are limitations in infrastructure, trained personnel, networking and coordination. Methods for obtaining data are often problematic, especially regarding the data on the use of antimicrobials. Studies well-designed, small-scale, such as research indicators in different contexts can be effective for understanding the overall situation and to identify priority areas for intervention (World Health Organization, 2012). The European Antimicrobial Resistance Surveillance Network (EARS-Net) is the continuation of the European Antimicrobial Resistance Surveillance System (EARSS). The objectives of EARS-Net are: i) to collect comparable and validated AMR data, ii) analyze temporal and spatial trends of AMR in Europe, iii) provide timely AMR data that constitute a basis for policy decisions, iv) encourage the implementation, maintenance and improvement of national AMR surveillance programs and iv) support national systems in their efforts to improve diagnostic accuracy in the surveillance chain by offering an annual External Quality Assessment (EQA) (Bronzwaer *et al.*, 1999).

1.1. Antibacterial agents

Antibiotics are usually classified in five functional groups, based on their structure and/or mode of action, including: i) inhibitors of cell wall synthesis, ii) inhibitors of protein synthesis, iii) inhibitors of membrane function, iv) anti-metabolites and v) inhibitors of nucleic acid synthesis (Jehl *et al.*, 2012). All of them, except the inhibitors of membrane function were relevant for this study (Figure 2).

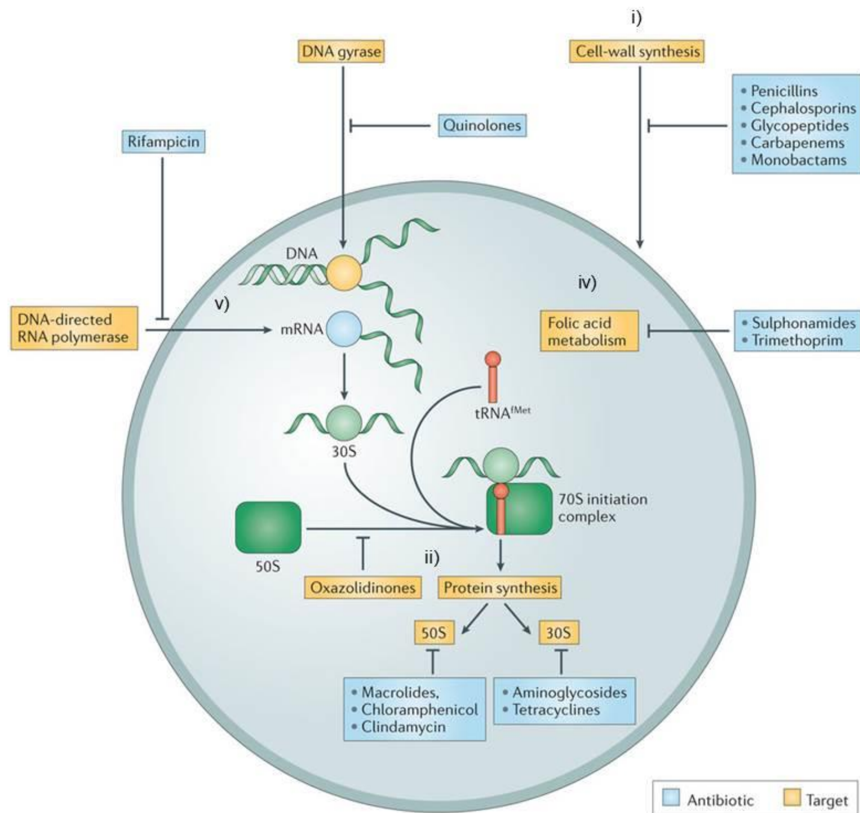

Figure 2: Representation of the site of action, targets of different classes of antibiotics (Lewis, 2013).

1.1.1. Inhibitors of cell wall synthesis

The β-lactam antibiotics are the most important class of inhibitors of cell wall synthesis. They are of the most relevant due to their high efficacy and low toxicity to animals, including humans. These antibiotics have in common one β-lactam ring in their chemical structure, which interferes with the synthesis of peptidoglycan bacterial cell wall (Quinteira, 1999). The β-lactams include the subgroups of penicillins (e.g. ampicillin and amoxicillin/clavulanic acid), cephalosporins (e.g. cefotaxime and ceftazidime), cephamycins (e.g. cefoxitin), monobactams (e.g. aztreonam) and carbapenems (e.g. imipenem).

1.1.2. Inhibitors of protein synthesis

The inhibitors of protein synthesis include the aminoglycosides, tetracyclines and phenicols.

i. Aminoglycosides

Aminoglycosides such as amikacin and streptomycin are multifunctional hydrophilic sugars that possess several amino and hydroxyl functionalities (Shakil *et al.*, 2008). Their bactericidal activity is concentration-dependent and they are more effective against susceptible bacterial populations that are rapidly multiplying. Their mechanism of action implies the entrance into the cell, followed by binding to 30S subunit of the bacterial ribosome, interfering with the initiation of peptide/protein formation. The transport system of these antibiotics is oxygen dependent which makes them harmless agents against anaerobic bacteria (Amaro, 2011).

ii. Tetracyclines

Tetracyclines (e.g. tetracycline) constitute a family of antibiotics containing a hydroxynaphthalene core, formed by four fused benzene rings (Amaro, 2011). Tetracyclines are bacteriostatic agents that bind to the 30S ribosomal subunit of the bacteria, inhibiting the binding of the aminoacyl-tRNA to the acceptor site of the mRNA-ribosome complex. Consequently, they prevent the addition of amino acids during the elongation (Atwater, 1950).

iii. Phenicols (Chloramphenicol)

Phenicols such as chloramphenicol bind irreversibly to 50S subunit of the bacterial ribosome, impairing the peptidyl transferase activity and preventing the amino acid transfer to growing peptide chains. Its use is prohibited in animals intended for food in some countries due to the fact that it can cause fatal aplastic anemia in humans (Amaro, 2011).

1.1.3. Anti-metabolites

An anti-metabolite, as suggested by the name, is a chemical that inhibits the use of a certain metabolite. An example is the antibiotic trimethoprim, which is a dihydrofolate reductase (DHFR) inhibitor. Thus, this antibiotic interferes with the production of tetrahydrofolate, required for the synthesis of nucleic acids and certain amino acids. It is normally administered with sulfamethoxazole, a combination often known as co-trimoxazole. Sulfamethoxazole is a sulfonamide and is a competitive inhibitor of the

bacterial enzyme dihydropteroate synthetase (DFPS). This enzyme catalyzes an earlier step of the folate synthesis pathway than DHFR (Atwater, 1950).

1.1.4. Inhibitors of nucleic acid synthesis

Quinolones (e.g. nalidixic acid) and fluoroquinolones (e.g. ciprofloxacin) are chemically synthesized antibiotics. They inhibit the synthesis of DNA because they prevent the function of DNA gyrase and topoisomerase IV. This interfers with the winding of bacterial DNA, preventing the cellular replication (Amaro, 2011).

1.2. General mechanisms of antibiotic resistance

In Gram-negative bacteria, there are several mechanisms by which bacteria can survive to the action of antibiotics (Figure 3). These include (Allen *et al.*, 2010):

a) Impermeable barriers: resistance due to the inaccessibility of the antimicrobial into the cell due to the presence of an impermeable membrane or due to the absence of the antibiotic target.

b) Multidrug resistance efflux pumps: presence of transport proteins that promote the expulsion of antibiotics from the cell to the outside environment. The efflux pumps may be specific to a substrate or may be able to carry structurally different compounds (Webber, 2002).

c) Resistance mutations: bacteria can acquire mutations in genes that cause the alteration of the target of the antibiotic, inhibiting its effectiveness. For instance, resistance to fluoroquinolones can be caused by mutations that alter the conformation of proteins involved in DNA replication.

d) Inactivation of the antibiotic: the antibiotics can be inactivated by covalent modification, such as that catalyzed by acetyltransferases acting on aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalyzed by β-lactamases acting on β-lactam antibiotics.

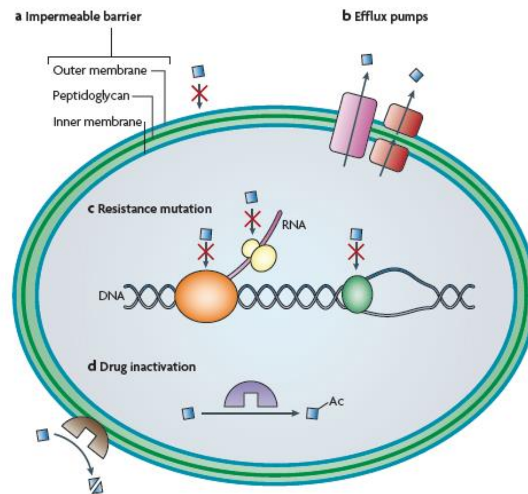

Figure 3: Schematic representation of the principal mechanisms of resistance to antibiotics (Allen *et al.*, 2010).

1.3. Movement of antibiotic resistance in the environment

The selection and dissemination of resistance in the environment is a very complex process. Major environmental reservoirs of resistance are probably created by human activities involving the use of antibiotics as well as waste disposal (Figure 4) (Davies & Davies, 2010). For instance, according to CDC (2013), up to 50% of all antibiotics prescribed in human medicine are not needed or are prescribed inappropriately. Also, some antibiotics are not completely metabolized, and are subsequently excreted via the urine or faeces even with some antimicrobial activity. In addition to clinics, they are widely used in the treatment of animals and in agriculture, contributing to the increase of selective pressure, which contribute to the selection of several resistance determinants and bacteria also in the environment (Dantas & Sommer, 2014). For instance, the microorganisms of the soil are considered the largest and most diverse reservoir of resistance. The spread of resistance in the environment can be influenced by physical factors such as those created by wind and watershed (Allen *et al.*, 2010). However, biological factors such as the acquisition of heterologous genes by lateral transfer largely facilitates the adaptive evolution of bacteria, in particular under strong selective pressures. This transfer of exogenous DNA in bacteria can be mediated by plasmids, phages, transposons, genomic islands, or by acquisition of free DNA by transformation (Lupo *et al.*, 2012).

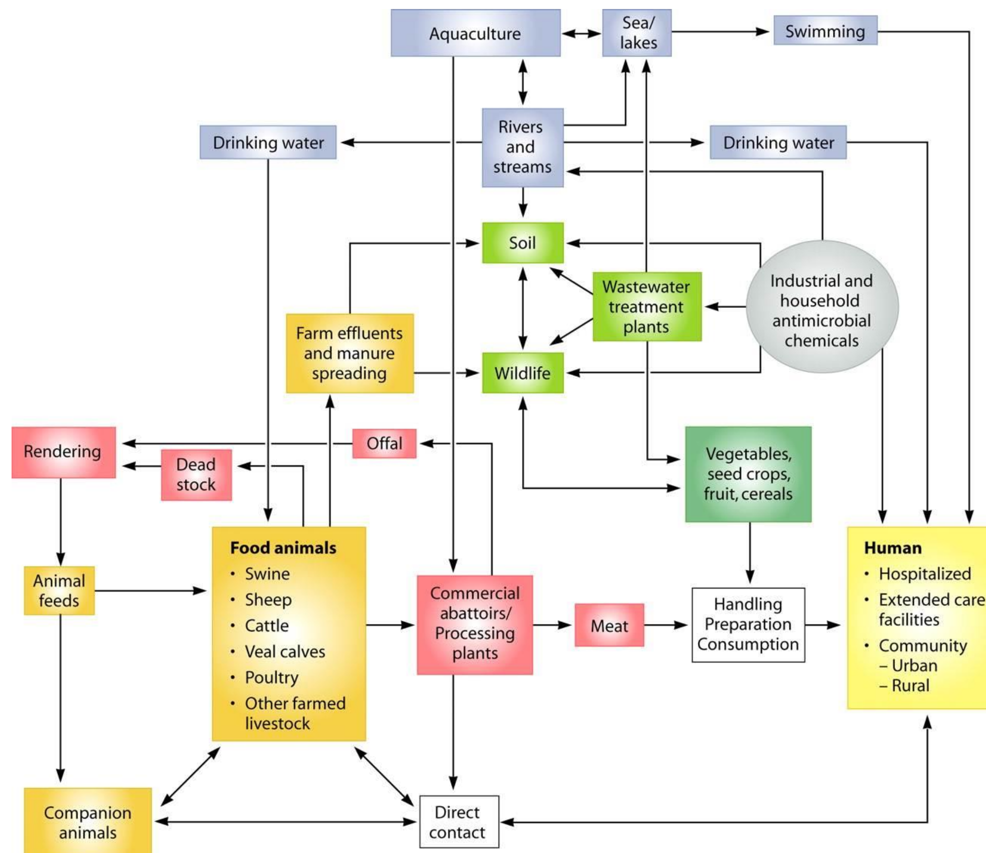

Figure 4: Schematic representation of factors involved in the dissemination of antibiotic resistance in the environment (Davies & Davies, 2010).

1.4. Antimicrobial resistance in wildlife

Wild animals provide a biological mechanism for the spread of antibiotic resistance genes (Figure 4). Usually, wild animals are not exposed to antimicrobial agents, but can acquire resistant bacteria due to contact with humans, other animals and the environment, where water polluted with faeces appear to be the main vehicle of contamination (Radhouani et al., 2014). Therefore, proximity to humans is predicted to influence the antibiotic resistance profile of the gut flora in wild mammals as it has been demonstrated in some studies (Allen *et al*., 2010). For instance, African baboons and apes that were in contact with humans carried more antibiotic-resistant enteric bacteria than those living in areas far away from anthropogenic influences (Rwego *et al*., 2008).

Wild birds are important reservoirs of antibiotic-resistant bacteria with the potential for long-distance dissemination. Birds and migratory waterfowl in particular, can travel great distances and inhabit a wide variety of environments, from agricultural lagoons to remote mountain lakes, and can potentially spread resistance genes along the way. Some

studies have associated the proximity to human activities with the increase of antibiotic-resistant bacteria associated with wild birds (Allen *et al.*, 2010). Also, a study in Berlenga Island from Portugal had identified gulls as major source of faecal pollution in coastal waters and potential vectors of human infection. Additionally, they were identified as an important vector of dissemination of antimicrobial resistance genes because of their association with human activities (Marta S Alves *et al.*, 2014). Moreover, resistant as well as multiresistant isolates of *E. coli* were detected in the normal flora of Arctic birds, one of the most remote areas on Earth (Sjölund *et al.*, 2008). In England, 90% of the bacterial isolates from mice and voles captured in rural England were resistant to β-lactam antibiotics (Gilliver *et al.*, 1999). On the other hand, faecal enterobacteria of wild elk, deer and voles in Finland have almost no resistance (Osterblad *et al.*, 2001). These results may be explained by the influence of human activities in each country (Allen *et al.*, 2010). Samples from red foxes collected in the North of Portugal were analyzed and 73% of the isolates presented resistance to at least one antimicrobial (Radhouani *et al.*, 2013).

1.5. Etiologic agents

As human populations grow and transform landscapes, contact with wildlife largely increases. Disease emergence has been an important consequence of this escalation in interaction, with the high number of emerging infectious diseases in humans arising from wildlife reservoirs. Zoonoses are infections or diseases transmissible between animals and humans, which can be acquired by ingesting contaminated food or water (e.g. *Salmonella* spp., *Campylobacter* spp., norovirus, STEC), or through direct contact with infected animals (e.g. *Salmonella* spp. and STEC) (EFSA & ECDC, 2014). According to the CDC, approximately 75% of the recently emerging infectious diseases affecting humans are of animal origin. Also, EFSA and ECDC reported a total of 5,550 food-borne outbreaks between 2005 and 2009, caused mainly by *Salmonella* spp., viruses and bacterial toxins. The transmission of pathogens from humans to wildlife is also a growing threat, which increases the management challenges of wildlife conservation.

1.5.1. *Escherichia coli*

E. coli is a Gram-negative bacillus and is a member of the Enterobacteriaceae family (the enteric bacteria), which is a facultative anaerobe commonly found in the intestinal tract of a wide variety of animals and humans. This intestinal bacterium can be easily disseminated in different ecosystems and its usefulness as a model for examining

the transmission of faecal microorganisms between humans and wildlife was already demonstrated (Silva *et al.*, 2011). In humans, *E. coli* is an important cause of urinary tract infections (UTIs), enteric infections and systemic infections. Genrelly, *E. coli* are often divided in intestinal pathogenic *E. coli* (causing infection in the gastrointestinal system) and extraintestinal pathogenic *E. coli* or ExPEC (cause infections outside the gastrointestinal system). The last incorporates the following variants: avian pathogenic *E. coli*, uropathogenic *E. coli* (UPEC), and those isolates responsible for septicemia and neonatal meningitis. The pathogenicity of ExPEC have been associated with the presence of several virulence genes (Pitout, 2012). *E. coli* is usually classified into four main phylogenetic groups (A, B1, B2 and D). Generally, the commensal strains are placed into the phylogenetic groups A and B1. Also, the *E. coli* strains causing extra-intestinal infections are known to mainly belong to group B2 and, to a lesser extent, group D. The intestinal pathogenic strains are usually assigned to groups A, B1 and D (Silva *et al.*, 2011). However, eight phylo-groups are now recognized: seven (A, B1, B2, C, D, E, F) belong to *E. coli sensu stricto*, whereas the eighth is the *Escherichia* cryptic clade I (Clermont *et al.*, 2013). Although there are some limitations to the use of *E. coli* as a model organism, it is still regarded as the global standard for detection of faecal contamination (Pesapane *et al.*, 2013).

1.5.1.1. Resistance in *E. coli*

In *E. coli* antibacterial resistance has been growing rapidly through mutations, frequently associated with fluoroquinolones resistance, or through the acquisition of mobile genetic elements involved in the resistance to third generation penicillins and cephalosporins. Resistance to third generation cephalosporins is mostly caused by extended spectrum beta-lactamases (ESBLs), which are able to hydrolyze many β-lactams (World Health Organization, 2014). The production of ESBLs by Enterobacteriaceae, specifically by *E. coli*, has caused a major concern in several countries, being frequently implicated in human infections. Especially worrisome is the significantly increase of resistance to third generation cephalosporins and combined resistance to at least three antimicrobial classes (multiresistant) reported by many countries during the period 2008–2011 (Figure 5). This is a serious public health concern since it severely limits the number of treatment alternatives for patients with life-threatening infections. Although EARS-Net data on ESBL production remain incomplete, a large percentage of third-generation cephalosporin-resistant *E. coli* was reported as ESBL-positive. Also, different reports have describing the spread of ESBL-positive *E. coli*

isolates in food products, in the intestinal microbiota of healthy food-producing animals and more recently in wild animals (Gonçalves *et al.*, 2012). Moreover, multidrug resistance was evaluated with *E. coli* isolates from farms in Michigan. The majority of *E. coli* isolates tested was sensitive to all antimicrobial agents tested, but 34% were resistant to one or two antimicrobial agents and 13% were resistant to three or more agents (Sayah *et al.*, 2005). Recently the first study reporting resistance of *E. coli* to antimicrobial agents in wild mammals (seagulls and deer) in Ireland was published. Resistance was found to penicilins, tetracyclines and rifamycins (Smith *et al.*, 2014). In another study involving *E. coli*, Iberian wolf samples were collected in Portugal and mostly showed resistance to tetracycline, ampicillin, streptomycin and co-trimoxazole. Their predatory and travelling habits might be a source of exposure of this species and could in that way explain the high rates of resistance that were found (A Gonçalves *et al.*, 2013).

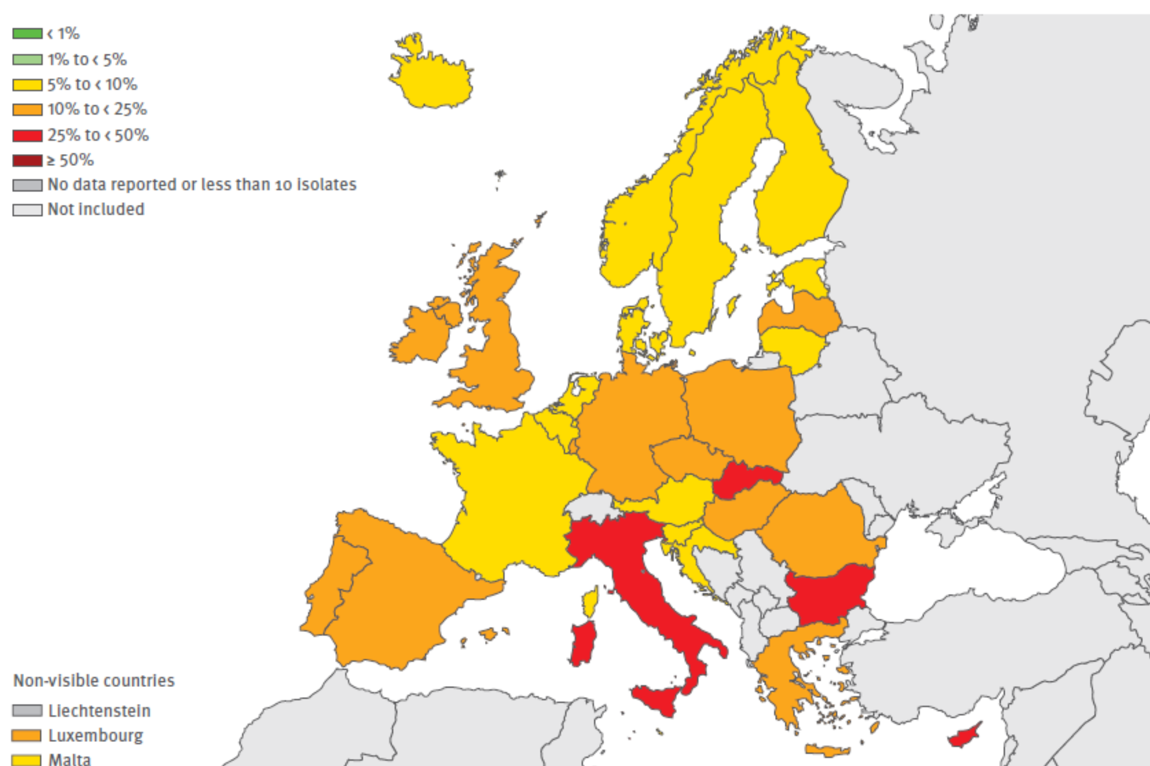

Figure 5: Percentage of *E. coli* isolates resistant to third-generation cephalosporins, by country in 2013 (European Centre for Disease Prevention and Control., 2014).

1.5.1.2. Shiga toxin-producing *E. coli*

E. coli has many different serotypes categorized into five major groups that are pathogenic to humans. These groups are designated according to virulence mechanisms: the first four are enterotoxigenic, enteropathogenic, enteroinvasive and enteroaggregative,

and the last one includes Shiga toxin–producing *E. coli* (STEC), which was previously known as enterohemorrhagic *E. coli* (WHO, 2011). STEC bacteria produce potent cytotoxins, called shiga toxins, which are composed by two subunits, A and B. The subunit A is the actual toxin that needs to interact with the subunit B (homopentamer) in order to bind to its cellular receptor the glycosphingolipid globotriaosylceramide (Gb3 or CD77; Figure 6) (Philpott & Ebel, 2002). Then, it is internalized by endocytosis and undergoes retrograde transport to the Golgi complex and then to the endoplasmic reticulum (ER) (4). In the ER, StxA encounters its target, the ribosome, inactivating it and causing inhibition of protein synthesis, leading to cell death by apoptosis (Figure 9) (Pacheco & Sperandio, 2012).

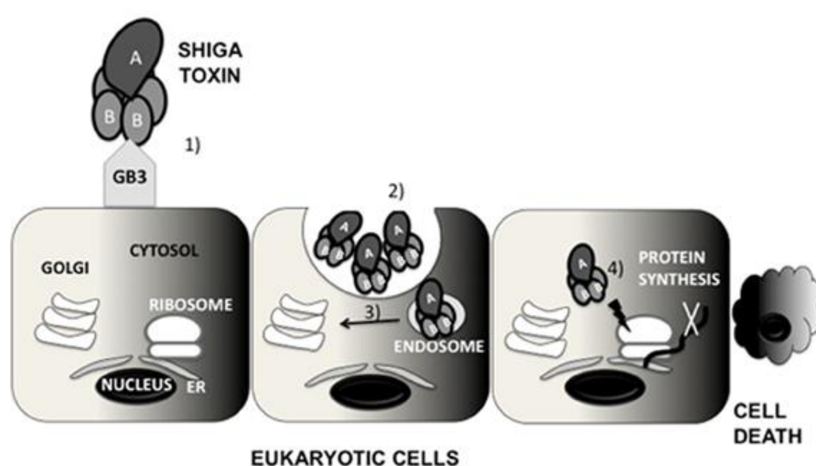

Figure 6: Mechanism of action of Shiga Toxin (Pacheco & Sperandio, 2012).

Infection with STEC may present a wide spectrum of clinical manifestations that can go from mild diarrhea to severe gastrointestinal and systemic diseases, such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (Sharma, 2006). The O157:H7 serotype is the better well-known STEC serotype and it is the principal cause of food-borne HUS in North America. Thus, STEC are frequently divided in the O157 and non-O157 strains. Many laboratories do not routinely screen and isolate non-O157 STEC, mainly because of the lack of selective media and/or immune-chemical reagents for the detection of other O-types than O157 (Cooley *et al.*, 2013). However, the number of infections caused by non-O157 STEC has increased since more laboratories are applying culture-independent tests to detect these strains (Gould *et al.*, 2013). These often imply the identification of the shiga-toxin genes, with emphasis on the StxA subunit. The *stx* family is divided in two main groups, the *stx1* and *stx2*. In the *stx1* family, the variants

stx1, *stx1c*, and *stx1d* were already described. The *stx2* family is composed by the variants *stx2c*, *stx2c2*, *stx2d*, *stx2e*, and *stx2f*. The Stx2 variants differ in their biological activity, immunological reactivity, and the receptor to which they bind. These binding properties allow them to target different cell types (Pacheco & Sperandio, 2012).

STEC infection has been associated with the consumption of contaminated foods, such as inadequately cooked ground beef, unpasteurized apple juice and cider, unpasteurized milk and other dairy products, including raw vegetables (Figure 7) (Gansheroff & O'Brien, 2000). Different animal species possess STEC strains as part of their gut flora. In fact, ruminants especially cattle have been identified as the major reservoir of STEC strains highly virulent to humans (Hussein, 2007; Miko *et al.*, 2009). Thus, the faeces of these animals are an important source of STEC strains for the environment. More recently, wildlife animals such as deer and migrating birds were also identified as an important source of STEC in the nature (Miko *et al.*, 2009). For instance, 49% of the samples belonging to wild or exotic mammals (including red and roe deer) from a study conducted in Belgium were positive for STEC strains (Piérard *et al.*, 1997).

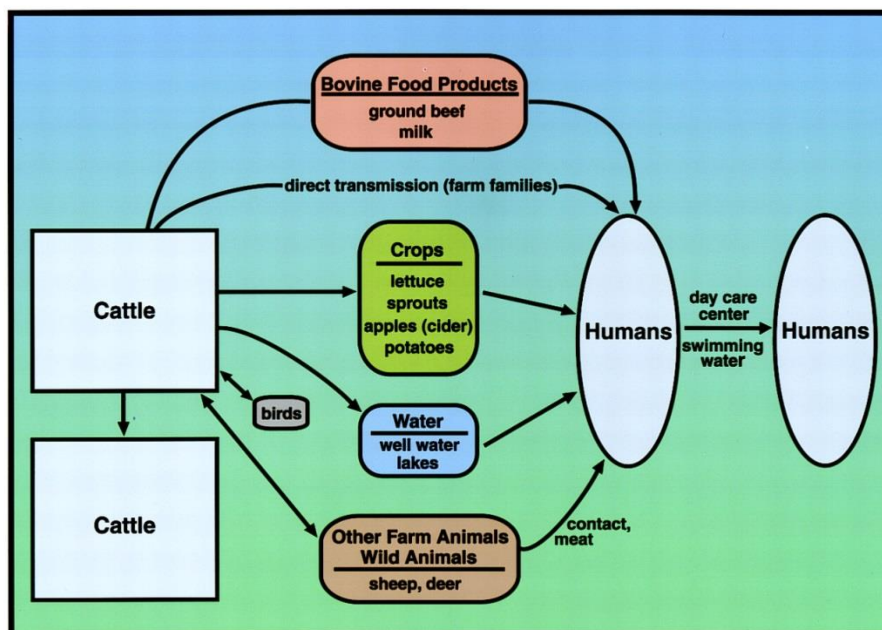

Figure 7: Model of the STEC transmission pathways from cattle to humans (Gansheroff & O'Brien, 2000).

1.5.2. *Salmonella* spp.

Salmonella spp. is commonly found in the intestines of healthy birds and mammals and is a bacterium that can cause salmonellosis in humans (European Food Safety Authority, 2014). In fact, it is the second most common cause of zoonotic diseases in humans, followed by *campylobacteriosis* (Botti *et al.*, 2010). During 2010, 99.020 cases of infections by *Salmonella* spp. were confirmed in Europe (Chiari *et al.*, 2013). A study developed in Portugal revealed that *Salmonella* spp. was responsible for 41.8% food-poisoning-related outbreaks in the period 1987–1991 in that country (Caleja *et al.*, 2011). The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori*. The first one includes 6 subspecies (subsp.): *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica*. Besides, over 2,500 serotypes of zoonotic *Salmonella* were already identified, most belonging to the subspecies *enterica* (Botti *et al.*, 2010). The three main serovars of *S. enterica* are Typhimurium, Enteritidis, and Typhi. Overall, considering all *Salmonella* infections in the EU, *S.* Enteritidis and *S.* Typhimurium are the serovars most frequently associated with human illness (EFSA & ECDC, 2014).

There are certain risk factors for human *salmonellosis*: infection may indirectly arise in agricultural areas from the contamination of vegetable products, through direct animal contact, during hunting, or directly from ingestion of contaminated meat or their products. Moreover, *salmonellosis* causes significant economic losses to the livestock industry. Actually, *Salmonella* species are able to infect a wide range of domestic and wild animal species and have been isolated from the intestinal content of white-tailed deer, rabbits and wild boars (*Sus scrofa*) (Chiari *et al.*, 2013). In a study designed to evaluate the prevalence of *Salmonella spp.* in faecal samples of wild boars and wild rabbits hunted in Northern Portugal, the results showed that 22% of the wild boars presented *Salmonella spp.* in their faeces (Vieira-Pinto *et al.*, 2011). Cattle seem to contribute to the *Salmonella* spp. prevalence in wild boar by introducing their own serotypes to the environment (Navarro-Gonzalez *et al.*, 2012).

Salmonella is also of public health problem because many strains are resistant to antimicrobial agents. In wildlife, the levels of antibacterial resistance in *Salmonella* spp. vary considerably, mainly depending on the host species, the bacterial species and the geographic location (Navarro-Gonzalez *et al.*, 2012). However, the prevalence of resistance, including ampicillin and co-trimoxazole, has increased in recent decades. The location of specific antibiotic-resistance genes on mobile genetic elements (such as plasmids and transposons) facilitates the transmission of resistance among bacteria, even among different species (Caleja *et al.*, 2011).

1.6. Ungulates in study

Ungulates can be excellent model species to investigate AMR because: i) their increase in number during the last decades, ii) they have a considerably wide home range when compared with small mammals, iii) they are unlikely to be treated with antibiotics, iv) they share their habitat with livestock and humans and v) they allow exploring AMR in a geographic gradient (Torres *et al.*, 2014). Thus, studies investigating the occurrence of AMR and potential pathogens in wild ungulates are urgently needed. Nevertheless, in Portugal, the information available is restricted to Northern populations (Poeta *et al.*, 2009). In this thesis, three ungulates will be the focus of study: wild boar, red deer and roe deer.

1.6.1. Wild boar

Wild boar (*Sus scrofa*) is a widely distributed ungulate whose success can be attributed to a variety of ecological features such as opportunistic omnivorous behavior and also high proliferative and adaptive capacity (Ferreira *et al.*, 2009). Wild boar is a species that is utilized for food and sport hunting throughout the world. They have been increasing their natural populations, which in some circumstances, leads to conflicts involving several sectors, including agriculture damages, conservation problems and health risks. The disease-related conflicts are especially relevant since wild boar was raised as a potential host for numerous pathogens (Acevedo *et al.*, 2014). Nowadays, this species are wide-spread throughout Portugal, with the exception of major urban areas and some parts of the coastline, they are also of ''low concern'' in terms of their national conservation status (Ferreira *et al.*, 2009).

1.6.2. Red deer

There are several variables capable of influencing the habitat use and selection by the red deer. Besides the land cover units, variables like vegetation productivity, water proximity, diversity of plants, distance to roads, distance to villages, altitude, aspect and slope are identified as key factors influencing red deer habitat use (Joana Alves *et al.*, 2014). In Portugal, red deer (*Cervus elaphus*) populations have increased in size and distribution over the last three decades (Santos *et al.*, 2011). One of the largest free-ranging populations is located on the Spanish border, in the Northeast of the country (*Montesinho* Natural Park). Red deer recolonization of this region dates from the early 1980s, when several animals dispersed naturally from *Sierra de la Culebra* Regional Hunting Reserve (Zamora, Spain) into the Portuguese territory (Santos *et al.*, 2011). The

presence of red deer in Lousã is an outcome of a reintroduction process that occurred from 1995 to 1999, with the release of 96 animals. Since then, the population has expanded geographically and demographically, with the occupancy of new territories (Joana Alves *et al.*, 2014).

1.6.3. Roe deer

The European roe deer (*Capreolus capreolus*) is the most abundant and widespread cervid species in Europe (Torres *et al.*, 2011). Within the distribution range, roe deer occurrence is influenced by a variety of factors including food availability, cover, human disturbance, terrain characteristics, climatic factors and predation (Torres *et al.*, 2012). Roe deer is a native species in the North of Portugal, where populations have always persisted. During the 90's a series of reintroductions took place in the centre of Portugal to increase prey availability for the endangered Iberian wolf, and in the south for touristic hunting grounds (Valente *et al.*, 2014).

1.7. Sampling locations

1.7.1. Montesinho Natural Park

The Montesinho Natural Park is one of the largest natural parks of the 12 existing in the country. Is situated in the region of Tras-os-Montes (Northeastern) covering the Northern part of the districts of Bragança and Vinhais. It was created in 1979 and contains about 240 species of vertebrates. It is a region with low human and cattle density (Cepo Verde Turismo Rural).

1.7.2. Lousã Region

The Lousã Mountain is located in the district of Coimbra transition to Leiria. The importance of biological and ecological richness that Lousã Mountain contains has been recognized nationally and internationally for its inclusion in the Natura 2000 network, concerning classified sites for possessing habitats and species of community interest (Fonseca *et al.*, 2009). In the 1990s, Lousã was enriched through the reintroduction of cervids, including deer and roe deer, with a view not only increased local animal biodiversity, as well as a hunting farm, framed in the sustainable management of their populations. This region has high human density and medium livestock density (Fonseca *et al.*, 2009).

1.7.3. Idanha-a-Nova

Idanha-a-Nova is a Portuguese village in the district of Castelo Branco, one of the largest townships of Portugal, however sparsely populated. Regarding the fauna, the presence of 277 vertebrate refers to the enormous wealth and faunal diversity. Herdade de Vale Feitoso in Penha Garcia was the collection site in this region, having an area of about 7,000 hectares fully fenced. It is a zone of tourist hunting and given the conditions of their habitat, especially favorable for the big game hunting, here we find the Corsican mouflon, red deer, fallow deer and wild boar. This zone has a low population density, but a high density of livestock (Núcleo Executivo de Idanha-a-Nova, 2005).

1.8. Objectives

Antimicrobial drugs have been widely used in human and veterinary medicine, with tremendous benefits to both human and animal health. The development of resistance to these medicines poses a serious public health threat. Monitor antimicrobial resistance in bacteria of clinical, foodborne and wildlife origin is extremely important. Therefore, studies investigating the antibiotic susceptibility of bacteria from wild ungulates are increasing. In Portugal, the information available is restricted to the Northern population. Thus, the present study focused on three species of wild ungulates inhabiting different geographical areas with different anthropogenic influences, from North to Central Portugal (Lousã region, Idanha-a-Nova and Montesinho). Thus the main objectives of the present study were:

i) To access the levels of antibacterial resistance occurring in *E. coli* strains collected from Portuguese wild ungulates faeces;

ii) Determine the occurrence levels of *Salmonella* spp. in Portuguese wild ungulates;

iii) Determine the occurrence levels of shiga toxin-producing *E. coli* (STEC) in Portuguese wild ungulates;

2. Material and Methods

2.1. Flowchart and methodologies followed

The experimental strategy applied in the present study after field collection of the samples is presented on Figure 8.

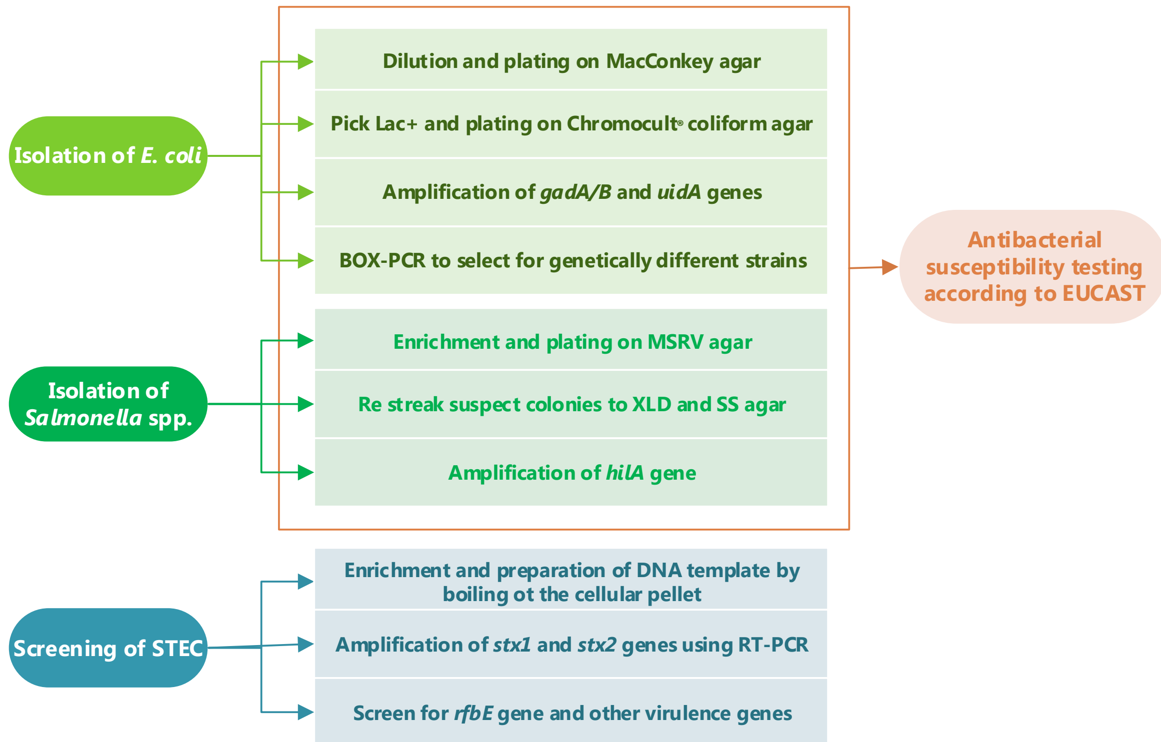

Figure 8: Schematic representation of the experimental strategy used in this work.

2.2. Origin and description of the samples

A total of sixty-seven faecal samples from different geographic locations were collected in Portugal by Wildlife Research Unit (UVS) of the University of Aveiro from October 2013 to April 2014. Samples of red deer (n=42) and roe deer (n=4) were collected from natural environments, when considered fresh. Samples of wild boar (n=21) were collected only from hunted animals and directly from the rectal area. The collection was performed using sterile recipients. The list of samples collection date, location and species are shown in Tables 10, 11 and 12 (Appendix 2). The samples were kept in a field cooler up to 2 h and afterwards stored at 4 °C until processed. The sampling locations (Figure 9) were i) Montesinho Natural Park (Bragança), a region with low human and cattle density, ii) Lousã, a region with high human density and medium livestock density and iii) Herdade de Vale Feitoso (Idanha-a-Nova), that is characterized by a low population density, but a high density of livestock. Once in the lab, 1 g of each faecal

sample was smashed and diluted in 10 mL of Buffered Peptone Water (BPW; Merck) under aseptic conditions. The composition of the culture media used is described in Appendix 1.

Figure 9: Map of Portugal with the collecting sites indicated.

2.3. Isolation and selection of *E. coli* isolates

Serial dilutions of each sample were prepared and cultured on MacConkey agar plates (Oxoid), overnight at 37 °C. Ten lactose-positive colonies with *E. coli* phenotype were randomly selected and subcultured on Chromocult® coliform agar (Merck), overnight at 37 °C. In this media, only the blue to violet coloured colonies were considered *E. coli* isolates, using *E. coli* ATCC® 25922 as the positive control.

2.3.1. Amplification of gadA/B (GAD) and uidA (GUD) genes

PCR with *gadA/B* and *uidA* genes is one of the several techniques used for genotypic confirmation of *E. coli.* To confirm that the blue to violet isolates in Chromocult® agar belong to the *E. coli* species, a PCR was performed targeting the genes mentioned

above. Glutamate decarboxylase (GAD) is an enzyme that catalyzes the decarboxylation of glutamate to GABA (gamma-Aminobutyric acid) and CO_2, and *uidA* gene codes for β-D-Glucuronidase (GUD). GAD and GUD have the ability to detect various strains of *E. coli*, and to discriminate between other species. Most of nonpathogenic *E. coli* strains are reported to contain GUD and most other bacteria containing GUD do not grow on media selective for coliforms. A genotypic assay for GAD should be expected to show greater quantitative sensitivity because it would actually target two highly homologous genes, *gadA* and *gadB*, that always occur in the genome of *E. coli*. *Shigella* spp. can also be positive in this assay, but these bacteria do not ferment lactose and therefore would not be selected in MacConkey screening (McDaniels *et al.*, 1996).

PCR amplification was performed in a final volume of 12.5 µl containing 3 mM MgCl2, 0.2 mM dNTPs, 1X Green GoTaq® Reaction Buffer, 0.3 pmol/µL of each primer (the sequence of the primers is shown in Table 1), 1 µl of template DNA, and 0.3U of GoTaq® Buffer (Promega). The DNA template was prepared by resuspending each colony in 100 µl sterile nuclease free distilled water and boiled at 95 °C for 10 min. The parameters of amplification were: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 45 sec and a final extension step at 72 °C for 10 min. The amplification products were separated by electrophoresis in a 1% agarose gel at a constant voltage of 120V.

After confirmation that the chosen isolates were *E. coli*, a maximum of three genetically different *E. coli* strains were selected to assess their respective antibacterial resistance profile.

Table 1: List of primers used in the PCR reactions for amplification of *gadA/B* and *uidA* genes.

Name	Sequence	Reference
gadA/B forward	5'-ACCTGCGTTGCGTAAATA-3'	(McDaniels *et al.*, 1996)
gadA/B reverse	5'-GGGCGGGAGAAGTTGATG-3'	
uidA forward	5'-CCAAAAGCCAGACAGAGT-3'	
uidA reverse	5'-GCACAGCACATCAAAGAG-3'	

2.3.2. BOX-PCR fingerprinting

The strains isolated from each sample were submitted to rep-PCR genomic fingerprinting (n=640). This method is based on the use of primers targeting naturally

occurring, highly conserved and repetitive DNA sequences present in multiple copies in bacteria (Lupski & Weinstock, 1992). Three families of repetitive sequences are normally used, including the 35-40bp repetitive extragenic palindromic (REP) sequence, the 124-127bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154bp BOX element (Versalovic *et al.*, 1994; Rademaker & De Bruijn., 1997). In this study, all the isolates were submitted to BOX-PCR. The DNA template was prepared by growing each *E. coli* isolate in 500 µl of LB medium (Merck) at 37 °C, overnight with aeration at 180 rpm. Each culture was boiled at 95 °C for 10 min and centrifuged at maximum speed for 5 min. The supernatant was removed and the pellet resuspended in 100 µl of sterile distilled water, before used in the PCR reaction. The amplification was performed in a final volume of 12,5 µl, containing 3 mM MgCl2, 0.2 mM dNTPs, 1X Green GoTaq® Reaction Buffer, 0.6 pmol/µL of BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'), 0.5 µl of template DNA and 0.3U of GoTaq® Buffer (Promega). The parameters for amplification were, initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 sec, 53 °C, 52 °C or 53 °C for 30 sec and 72 °C for 8 min and a final extension step at 72 °C for 10 min. The products of amplification were separated by electrophoresis in a 1.5% agarose gel at a constant voltage of 120V during 1 h and 30 min, and the gels stained with ethidium bromide were visualized under the UV light. The fingerprints obtained were analyzed with GelCompar II 5.0 program (Applied Maths, Kortrijk, Belgium), where gel images were normalized and bands were identified. Clonal relationship was analyzed using Dice similarity coefficients and the UPGMA clustering method.

2.3.3. Conservation of bacterial isolates

After the selection of the 152 isolates, a glycerol stock of each of them was prepared by growing the colonies in 400 µl of LB medium at 37 °C with aeration. After overnight growth, 200 µl of 45% sterile glycerol were added and stored at -80 °C.

2.4. Detection of *Salmonella* spp.

The detection of *Salmonella* spp. was performed according to ISO 6579:2002 Annex D (International Organization for Standardization, 2007), recommended by the European Union Reference Laboratory. The faecal samples diluted in BPW that were used for preparing serial dilutions for the isolation of *E. coli*, were incubated at 37 °C at 180 rpm. After 20 h of growth, three drops of each culture (100 µL) were inoculated on

Modified semisolid Rappaport-Vassiliadis (MSRV; Oxoid) agar plates and grown at 41.5 °C for 24 h. The plates without or with doubtful growth of *Salmonella* spp. were incubated for an additional 24 h. The samples suspected of *Salmonella* spp. growth were plated on SS agar (Merck) and Xylose Lysine Desoxycholate agar (XLD; Oxoid) and incubated at 37 °C for 24 h. In the SS agar, translucent or translucent with black centre colonies were selected as *Salmonella* spp. isolates, whereas in the XLD agar the colonies selected were those with red or red with black center. *Salmonella* Enteritidis ATCC® 13076™ was used as the positive control.

2.4.1. Amplification of *hilA* gene

PCR targeting the *hilA* gene is one of the several techniques employed for genotypic confirmation of *Salmonella* spp. Thus, to confirm that the selected isolates on SS agar and XLD agar belong to *Salmonella* spp., a PCR targeting this gene was performed. During the process of *Salmonella* infection, the presence of invasive genes (such as gene *hilA*) is required for bacterial entry into host cells, and many of these genes are encoded in SPI1 (*Salmonella* Pathogenicity Island 1). Thus, this method utilizes primers against *hilA* gene which is conserved in all *Salmonella* serovars, and that is absent in *Salmonella* close relatives (pathogenic and non-pathogenic) (Pathmanathan, 2003). PCR amplification was performed in a final volume of 12.5 µl containing 3 mM MgCl2, 0.2 mM dNTPs, 1X Green Master Mix, 0.3 pmol/µL of each primer (Table 2), 1 µl of template DNA, and 0.6U of NZYTaq DNA polymerase. The DNA template was prepared by resuspending each colony in 25 µl sterile nuclease free distilled water and boiled at 95 °C for 10 min, then the samples were centrifuged at 7300 x g for 5 min and the supernatant was used (Marathe *et al.*, 2012).

The amplification conditions were: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 66 °C for 30 sec and 72 °C for 45 sec and a final extension step at 72 °C for 5 min. The amplification products were separated by electrophoresis in a 1.5% agarose gel at a constant voltage of 120V. The isolates that were confirmed as belonging to *Salmonella* spp. were conserved in the same form that is described in Section 2.3.3.

Table 2: List of primers used in the PCR reactions for amplification of *hylA* gene.

Name	Sequence (5’->3’)	Reference
hilA forward	TTAACATGTCGCCAAACAGC	(Marathe *et al.*, 2012)
hilA reverse	GCAAACTCCCGACGATGTAT	

2.5. Screening and selection of STEC isolates

2.5.1. DNA template preparation for PCR

The enrichment culture from each sample that was prepared for the screening of *Salmonella* spp. (section 2.4. Detection of *Salmonella* spp.*)* was used for this protocol. 1 mL of this culture was centrifuged for 2 min at 9100 x g. The pellet was resuspended in 1 mL of sterile nuclease free distilled water and 100 µL of this sample was transferred to a 0.2 mL tube. Subsequently, the samples were heated to 80 °C for 5 min, followed by 20 min at 100 °C in a thermocycler. Then, the samples were centrifuged for 10 min at 4600 x g to remove cell debris and the supernatant was stored at -80 °C until its use as DNA template (Cooley *et al.*, 2013).

2.5.2. Screening of STEC in faecal samples by PCR

All the samples obtained were screened for the presence of shiga-toxin *E. coli* (O157:H7 and non-O157:H7) by real-time PCR amplification targeting the *rfb*E, *stx1* and *stx2* genes. Prior to the screening, all the samples were submitted to real-time PCR using primers for amplification of 16S rRNA genes (Table 3) to ensure that the sample by itself was not inhibiting the reaction. With this approach, it was established that a dilution of 1:10 should be used in the PCR reactions. The *rfbE* gene was amplified alone, whereas a multiplex was used to detect the *stx1* and *stx2* genes (Cooley *et al.*, 2013). The *E. coli* E242M (non-O157 STEC: *rfbE*(-), *stx1*(+*)*), and *E. coli* E176V, (O157:H7 STEC: *rfbE*(+*)*, *stx2*(+)) strains were kindly provided by Neiker tecnicalia (Spain) and their DNA was used as positive control. RT-PCR amplifications were performed in a final volume of 10 µl containing 1X SsoFast™ EvaGreen® Supermix, 0.3 pmol/µL of each primer (Table 3), 1 µl of template DNA. The parameters of amplification were: incubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 min, 60 °C for 45 sec. The samples were considered positive if the Ct values for *16S* and *stx* genes were <21 and <40, respectively, and also if the melting temperature of the amplicon was in the range of 75.4 ºC - 78.6ºC (*rfbE*), 77.4 ºC - 77.8 ºC (*stx1*) and 77.4 ºC – 81.6 ºC (*stx2*).

Table 3: List of primers used for STEC screening by Real-Time PCR.

Name	Sequence (5'->3')	Reference
stx1_forward	CATCGCGAGTTGCCAGAAT	(Cooley et al., 2013)
stx1_reverse	TCCCACGGACTCTTCCATCT	
stx2abc_forward	GGACCACATCGGTGTCTGTTATT	
stx2abc_reverse	CCCTCGTATATCCACAGCAAAAT	
rfbE_forward	TCAAAAGGAAACTATATTCAGAAGTTTGA	(Sharma, 2006)
rfbE_reverse	CGATATACCTAACGCTAACAAAGCTAA	
16S rRNA_forward	GGGTTGCGCTCGTTGC	(Zhu *et al.*, 2013)
16S rRNA_reverse	ATG GYT GTC GTC AGC TCG TG	

2.5.3. Amplification of *rfbE*, *stx1, stx2, ehx* and *eae* genes in *E. coli* isolates

The *E. coli* strains recovered from the faecal samples with a positive result for the presence of STEC (according to section 2.5.2) were submitted to PCR targeting the *rfbE*, *stx1* and *stx2* genes. PCR amplifications were performed in a final volume of 25 µl containing 3 mM MgCl2, 0.2 mM dNTPs, 1X Green Master Mix, 0.3 pmol/µL of each primer (Table 4), 1 µl of template DNA, and 0.6U of NZYTaq DNA polymerase. The parameters of amplification were: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 sec, annealing temperature of 54 ºC (*stx1* and *rfbE*) and 58 ºC (*stx2*) for 30 sec and 72 °C for 45 sec and a final extension step at 72 °C for 5 min. The amplification products were separated by electrophoresis in a 1.5% agarose gel at a constant voltage of 120V. In the case of positive amplification, the PCR products were purified and sent for sequencing reaction. Subsequently, the presence of other virulence genes such *eae* and *ehxA* was investigated in the *E.coli* isolates identified as STEC. The reaction was performed as abovementioned, where the primers targeting the 16S rRNA, *eae* and *ehxA* genes (Table 4) were used simultaneously, using the annealing temperature of 55 °C.

Table 4: List of primers used for the identification of STEC and for other pathogenicity genes in *E. coli* isolates.

Name	Sequence (5'->3')	Reference
stx1_B_fw	GTTGCGAAGGAATTTACC	This study
stx1_B_rv	ATTTTATTGTGCGTAATCCC	
stx2_F4	GGCACTGTCTGAAACTGCTCCTGT	(Persson *et al.*, 2007)
stx2_R1	ATTAAACTGCACTTCAGCAAATCC	
stx2_R1e_f	TAAACTTCACCTGGGCAAAGCC	
*rfbE*_fw	CAGGTGAAGGTGGAATGGTTGTC	(Bertrand & Roig, 2007)
*rfbE*_rv	TTAGAATTGAGACCATCCAATAAG	
eae_forward	CATTATGGAACGGCAGAGGT	(Bai *et al.*, 2010)
eae_reverse	ACGGATATCGAAGCCATTTG	
ehxA_forward	TCTGTATCTGCGGGAGTTAG	(Son *et al.*, 2014)
ehxA_reverse	CAACGTGCTCAAACATAGCC-	

2.6. Antibiotic susceptibility testing

Antimicrobial susceptibility testing was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, using *E. coli* ATCC® 25922 as quality control (EUCAST, 2013).

2.6.1. Preparation of inoculum

The isolates were grown in LB medium (Merck) at 37 °C, overnight, with aeration at 180 rpm. On the following day the optical density at an absorbance of 625 nm was measured, and the cultures were adjusted to a final density of 0.1 in NaCl (0.9%).

2.6.2. Preparation of plates and application of disks

Preparation of Mueller Hinton Agar (MHA; Merck) plates was performed by pouring the medium into sterile Petri dishes (approximately 25 mL in a 90 mm circular plate and 71 mL in a 150 mm circular plate) to a height of 4 mm +- 0.5 mm. A sterile swab was dipped in the previously prepared inoculum and spread in three different directions on the MHA plates. Subsequently, the disks of antibiotics were applied with an Oxoid™ Antimicrobial Susceptibility 12-Place Disc Dispenser and with an Oxoid™ Antimicrobial Susceptibility 8-Place Disc Dispenser. The plates were incubated at 37 °C. After 16-20h of

growth, the inhibition diameters were examined and measured. The isolates were classified as resistant (R), intermediate resistant (I) or sensitive (S) according to the breakpoints established by the EUCAST (Clinical and ECOFFs) or CLSI (Table 5).

Table 5: List of antibiotics used, respective concentration, respective ECOFFs and clinical breakpoints according to EUCAST or CLSI.

Antibiotic	Concentration (ug)	ECOFF (mm)	Clinical breakpoints (mm)	
			S (≥)	R(<)
Ampicillin	10	14	14	14
Amoxicillin/clavulanic acid	20+10	19	19	19
Cefoxitin	30	19	19	19
Cefotaxime	5	23	20	17
Ceftazidime	10	22	22	19
Aztreonam	30	26	24	21
Imipenem	10	24	22	16
Amikacin	30	18	16	13
Streptomycin	10	__	15 (CLSI)	12 (CLSI)
Nalidixic Acid	30	19	19 (CLSI)	15 (CLSI)
Ciprofloxacin	5	25	22	19
Co-trimoxazole	1.25+23.75	16	16	13
Tetracycline	30	__	15 (CLSI)	12 (CLSI)
Chloramphenicol	30	17	17	17

3. Results and Discussion

3.1. Isolation and selection of *E. coli* isolates

A total of 67 faecal samples were collected from wild ungulates (wild boar, red deer and roe deer) inhabiting in the areas of Lousã (76.1%), Idanha-a-Nova (19.4%) and Montesinho (4.5%). *E. coli* was detected in 96% of the samples (n=64) (Figure 10). No *E. coli* isolates were recovered in three of the faecal samples obtained from red deer. More specifically, 60.9% of the positive samples were from red deer, followed by 32.8% from wild boar and 6.3% from roe deer (Figure 11). In this study, the percentage of faecal samples that allowed the isolation of *E. coli* strains was similar to that obtained with Iberian wolf in Portugal where a recovery of 82% was achieved, but considerably higher that isolates recovered from wild birds in Azores Archipelago (53%), red foxes from North of Portugal (42%) and Iberian lynx from South Spain (60%) (A Gonçalves *et al.*, 2013; Alexandre Gonçalves *et al.*, 2013; Radhouani *et al.*, 2013; Santos *et al.*, 2013).

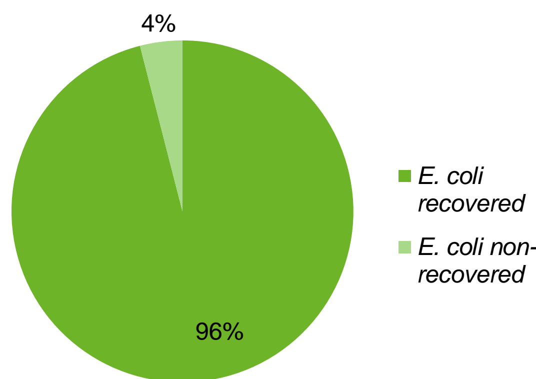

Figure 10: Percentage of the faecal samples from which *E. coli* were isolated.

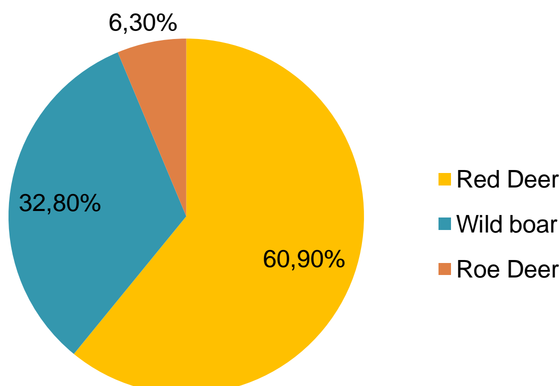

Figure 11: Distribution of the positive faecal samples according to the animal species of origin.

A total of 640 randomly selected colonies was subjected to rep-PCR fingerprinting with primers targeting the BOX-element (Figure 12). This analysis allowed the selection of 3, 2 or only 1 genotipically different *E. coli* isolates in 58%, 22% and 20% of the samples, respectively. So, a total of 152 *E. coli* strains were submitted to antibacterial susceptibility testing. From these, 59% were obtained from red deer (n=90), 36% from wild boar (n=55) and 5% from roe deer (n=7).

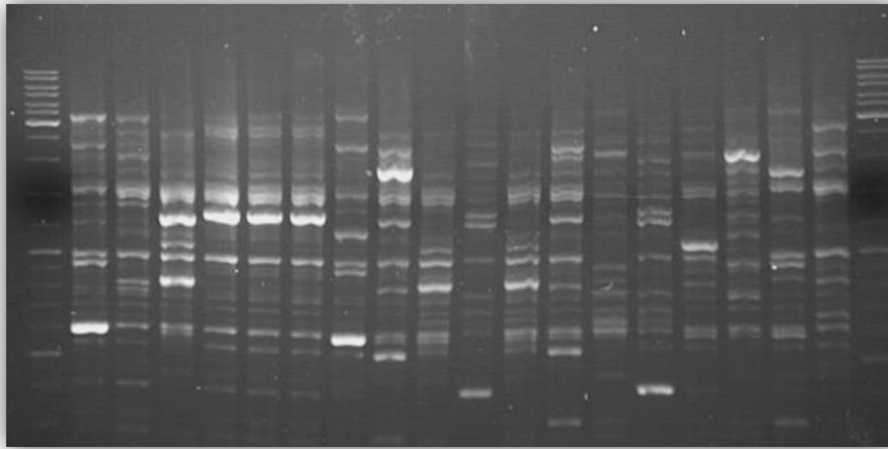

Figure 12: Example of a BOX-PCR fingerprint obtained in this study. The first and last lanes contain the DNA marker GeneRuler 1000bpplus (Thermo).

3.2. Presence of *Salmonella* spp.

Healthy wildlife animals can carry potential pathogenic bacteria, such as *Salmonella* spp, to humans over long periods of time (Navarro-Gonzalez *et al.*, 2012). Therefore, all the 67 faecal samples of this study were screened for the presence of *Salmonella* spp. After the cultivation in MSRV medium, 22 samples were positive or doubtful for this genera. From these, 14 samples presented suspicious colonies in SS and XLD agar. However, when these fourteen samples were subjected to PCR amplification, only one isolate was positive for the presence of the *hilA* gene. Accordingly, *Salmonella* spp. was identified in 1.5% (n=1) of the samples. The isolate was obtained from a wild boar's stool collected from the Lousã area. Similar results were obtained in another study from wild mammals and birds in North-western Italy (4,3%) (Botti *et al.*, 2010). Considering only the wild boar population screened, *Salmonella* spp. was present in 5% of the samples. A similar percentage was reported by Navarro-Gonzalez *et al.* in a study with

wild boars from Barcelona (Nora Navarro-Gonzalez *et al.*, 2013). Yet, this percentage is lower than that observed in wild boars from Northeastern Spain (17.5%) and from Northern Italy (25%) (Navarro-Gonzalez *et al.*, 2012; Chiari *et al.*, 2013). Wild boars can be exposed to innumerous sources of *Salmonella* spp., including mice and birds (Navarro-Gonzalez *et al.*, 2012). The isolated strain (J11) showed susceptibility to all of the antibiotics tested (considering both clinical breakpoints and the few epidemiological cut-offs available). In Northeastern Spain a study with wild boar reported low resistance values (3%) of *Salmonella* isolates, exhibiting resistance to ciprofloxacin, nalidixic acid, co-trimoxazole, streptomycin and chloramphenicol.

3.3. Antimicrobial susceptibility testing of *E. coli* isolates

In this study, the inhibition zone diameters (IZDs) of the 153 isolates (152 from *E. coli* and 1 from *Salmonella* spp.) were interpreted according to EUCAST clinical breakpoint values. However, EUCAST does not provide criteria for streptomycin, nalidixic acid and tetracycline. Therefore, for these antibiotics, CLSI breakpoints were used. The evaluation of the antibacterial resistance levels exhibited by the *E. coli* strains collected from Portuguese wild ungulates was performed according to i) clinical breakpoints and ii) epidemiological cut-offs. This strategy was chosen since the majority of the studies available in the literature consider only the clinical breakpoints. However, the application of ECOFFs is emerging and will be fundamental for resistance surveillance studies in the future.

3.3.1. Clinical Breakpoints

Our results showed that the majority (83.5%) of the *E. coli* isolates were susceptible to all the antimicrobial agents tested. Consequently, only 16.5% of the strains were resistant to at least one of those agents. More specifically, resistance was detected for ampicillin (10%), tetracycline (9%), streptomycin (5%), co-trimoxazole (4%), amoxicillin/clavulanic acid (2%) and cefoxitin (1%). Moreover, intermediate resistance was observed for streptomycin (11%), ceftazidime (6%) and aztreonam (1%). All the isolates were susceptible to cefotaxime, imipenem, amikacin, nalidixic acid, ciprofloxacin and chloramphenicol (Figure 13).

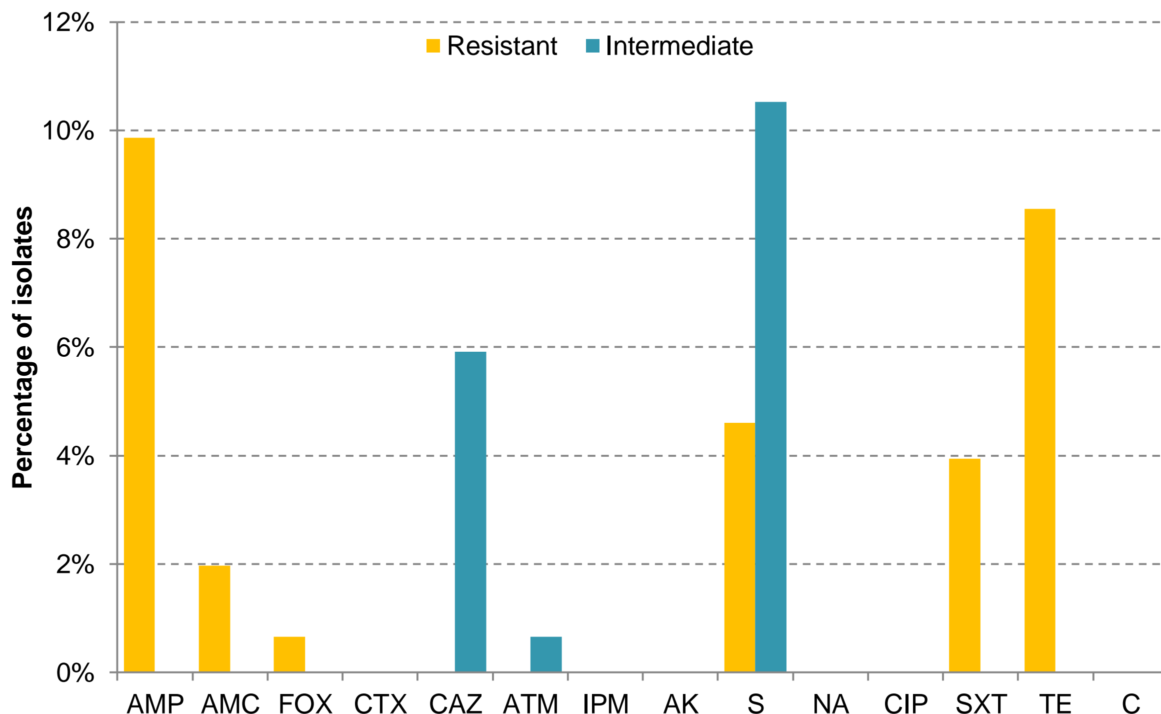

Figure 13: Percentage of resistant and intermediary *E. coli* isolates identified in this study according to clinical breakpoints (considering the total number of isolates). AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; S = Streptomycin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; TE = tetracycline; C = chloramphenicol.

Thus, the isolates collected from the Portuguese wild ungulates were mainly resistant to ampicillin, tetracycline, streptomycin and co-trimoxazole. Ampicillin is a β-lactam antibiotic that is amongst the most clinically important antimicrobial agents in both human and veterinary medicine. Additionally, tetracycline is commonly used as a first-line antibiotic for many different species of domestic animals. Similar results were found for wild small mammals living in a Canadian swine farm, where resistance was found to ampicillin, amoxicillin/clavulanic acid, cefoxitin, streptomycin, co-trimoxazole, tetracycline and chloramphenicol (Allen *et al.*, 2011). The highest levels of resistance in a study involving *E. coli* strains from various species of wild animals in Portugal were also observed for tetracycline (35%), streptomycin (22%), ampicillin (22%) and co-trimoxazole (19%) (Costa *et al.*, 2008). The same study described resistance to quinolones and chloramphenicol, which was not observed for the strains herein analyzed. Therefore, it appears that resistance to ampicillin, tetracycline and streptomycin should be expected when analyzing strains isolated from wild animals. However, the resistance levels can be distinct. For instance, in the *E. coli* isolates, which are subject of the present study, the percentage of resistance against these three antibiotics was 5 to 2 times lower than the

described in the other two studies abovementioned. In a study performed with faecal *E. coli* isolates from Iberian lynx, high percentages of resistance were observed to tetracycline (33%), streptomycin (28%), nalidixic acid (28%) and co-trimoxazole (22%), but unlike the animals of the present study, antibiotics are given to the Iberian lynx during management protocols (Alexandre Gonçalves *et al.*, 2013).

In theory, wild animals are not in contact with antimicrobial agents, since they are not submitted to therapy. However, antibiotics are constantly unloaded into the environment, as a result of their use in the treatment of human infections and their wide application in the veterinary and agricultural settings (Martínez, 2008). Moreover, environmental microorganisms are themselves producers of the majority of the antibiotics marketed today. Interestingly, the antibiotics with higher microbial resistance percentages identified (tetracyclines, penicillins and aminoglycosides) belong to the three classes of compounds with more sales in the Portuguese veterinary field, according to what was reported in 2011 by DGAV ((DGAV) Direcção-geral de alimentação Veterinária, 2011). Also, and according to the last European Medicine Agency (EMA) report (European Medicines Agency, 2013), tetracyclines and penicillins are the classes of antimicrobials mostly used for food-producing animals in Europe.

Considering each of the species, higher levels of resistance were generally obtained for wild boar, while the isolates originating from roe deer (n=7) were susceptible to all the antimicrobials tested. Specifically, the *E. coli* strains recovered from wild boar (n=55) were resistant to ampicillin (22%), tetracycline (11%), co-trimoxazole (9%), streptomycin (7%) and amoxicillin/clavulanic acid (2%). On the other hand, the *E. coli* isolates of red deer showed the highest percentage of resistance to tetracycline (8%) followed by ampicillin (3%) and streptomycin (3%). Resistance to amoxicillin/clavulanic acid (2%), cefoxitin (1%), and co-trimoxazole (1%) was also identified in the same population (Figure 14). The *E. coli* isolates from wild boar showed higher percentages of resistance than the isolates of red deer, except for amoxicillin/clavulanic acid and cefoxitin. In our study, 25% of the isolates from wild boar and 12% of the isolates from red deer were resistant to at least one of the antibiotics tested. In a wild boar population of central Europe, the percentage of strains with this phenotype was lower (6%) (Literak *et al.*, 2010). In another study with red deer from Stelvio National Park (Italy), 14% of the isolates collected showed resistance to at least one antimicrobial agent (Caprioli *et al.*, 1991). These last results are more in line with what was observed in the present study. No resistant isolates were identified from roe deer. Most likely, this result was due to the small sample size (n=4) and small number of isolates analyzed (n=7). In Portugal, the

information available for antibacterial resistance in wild ungulates is limited to wild boar in the North region of the country. Nonetheless, in that study the focus was in cefotaxime resistant *E. coli* (10% of the population) and the antibacterial resistance profile of the susceptible population was not evaluated (Poeta *et al.*, 2009).

The differences observed in the resistance levels of wild boar and red deer strains can probably be explained by their distinct phenology. Wild boars are scavengers omnivorous that come close to farms, consuming animal waste and even human garbage, while red deer are herbivores adapting their diet to what is available (Nora Navarro-Gonzalez *et al.*, 2013). Therefore, wild boars are in contact with areas highly influenced by anthropogenic activities, which implies a greater exposure to antibacterials as well as to bacterial strains of human origin.

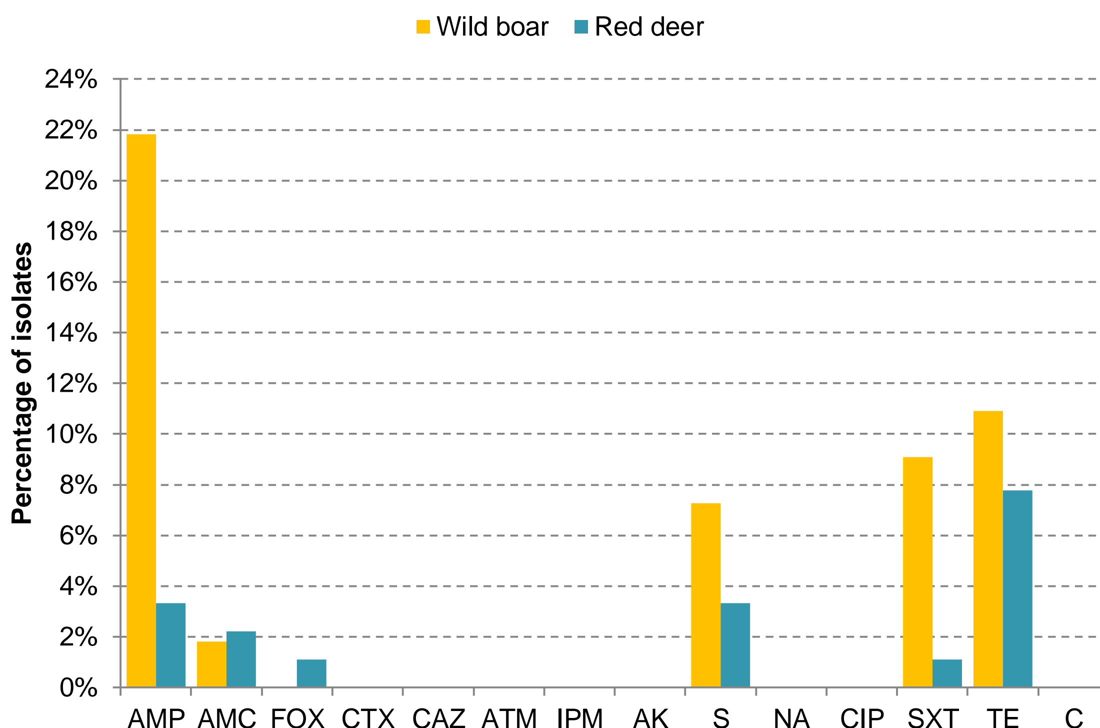

Figure 14: Percentage of resistant *E. coli* isolates from wild boar and red deer independently (roe deer was not included because no resistant isolates were identified). AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; S = Streptomycin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; TE = tetracycline; C = chloramphenicol.

If the geographical area is considered (Figure 15), this study showed that *E. coli* isolates resistant to streptomycin and co-trimoxazole were all recovered from animals living in Lousã area. On the other hand, resistance to cefoxitin was only detected in isolates with origin in Idanha-a-Nova. The strains isolated from wild ungulates from Montesinho only exhibited resistance to tetracycline. The comparison of the resistance

levels based on the geographical origin is difficult to perform due to the discrepancy in the number of isolates that were analyzed from each region (Lousã: n=110; Idanha-a-Nova: n=33; Montesinho n=9).

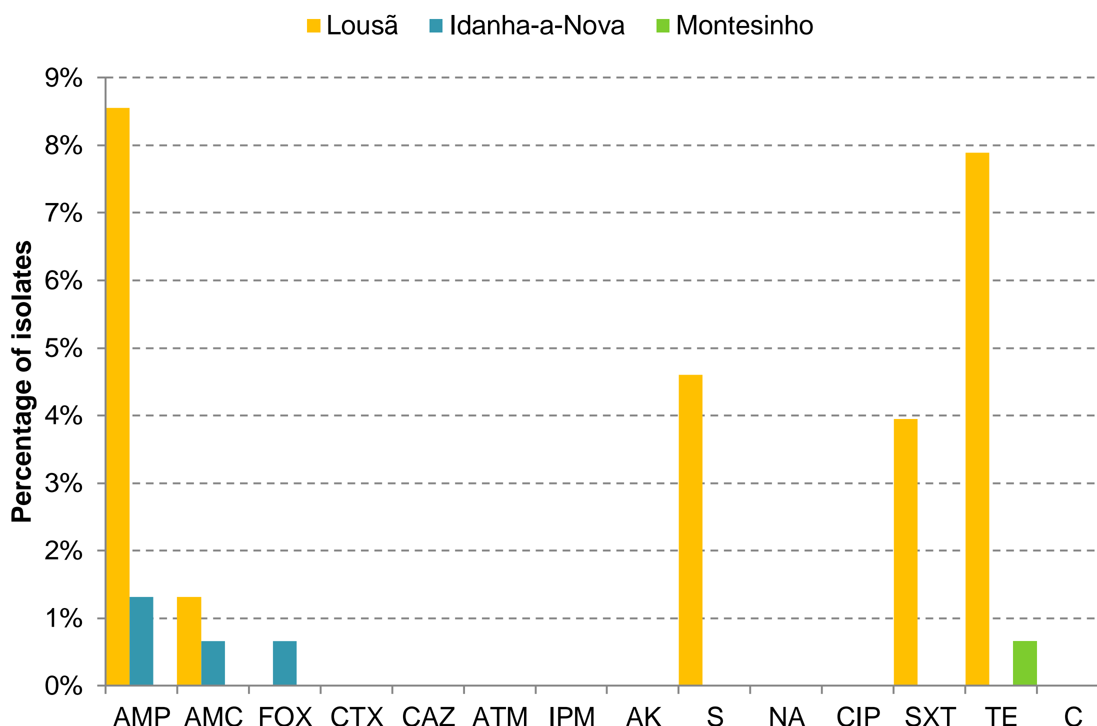

Figure 15: Percentage of resistant *E. coli* isolates based on their geographical origin. AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; S = Streptomycin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; TE = tetracycline; C = chloramphenicol.

3.3.2. Epidemiological cut-offs (ECOFFs)

The phenotypic antimicrobial susceptibility testing must have interpretation criteria so that the results are qualitative. These qualitative values can be based on clinical criteria or microbiological criteria. The last intend to distinguishing isolates with and without phenotypically detectable acquired resistance mechanisms and are designated as epidemiological cut-off values (ECOFFs) (Sjölund *et al.*, 2009). According to the EUCAST definition, a microorganism is defined as wild type (WT) by the absence of acquired and mutational resistance mechanisms to a determined antimicrobial. Thus, by application of an ECOFF to a bacterial MIC distribution, microorganisms can be classified as wild type or non-wild type. In this way, it is considered that ECOFFs are not changed by sampling time, source (human, animal, environmental) or geographical origin (Brown, 2011). ECOFFs have no obvious relationship to the clinical breakpoints, which are determined by

committees to be clinically relevant (Kahlmeter, 2011). Since the samples had no clinical but environmental origin, the results were also analyzed according to the ECOFFs.

For *E. coli*, there is no definition of these cut-offs for disk diffusion for tetracycline and streptomycin and therefore, results for these two antibiotics will be discussed separately and later in this document. Thus, 82% of the *E. coli* isolates showed IZDs within the wild type values for all the tested antimicrobials. Non-wild type phenotypes were identified for ampicillin (10%), ceftazidime (6%), co-trimoxazole (4%), amoxicillin/clavulanic acid (2%) cefotaxime (2%), aztreonam (1%) and cefoxitin (1%) (Figure 16).

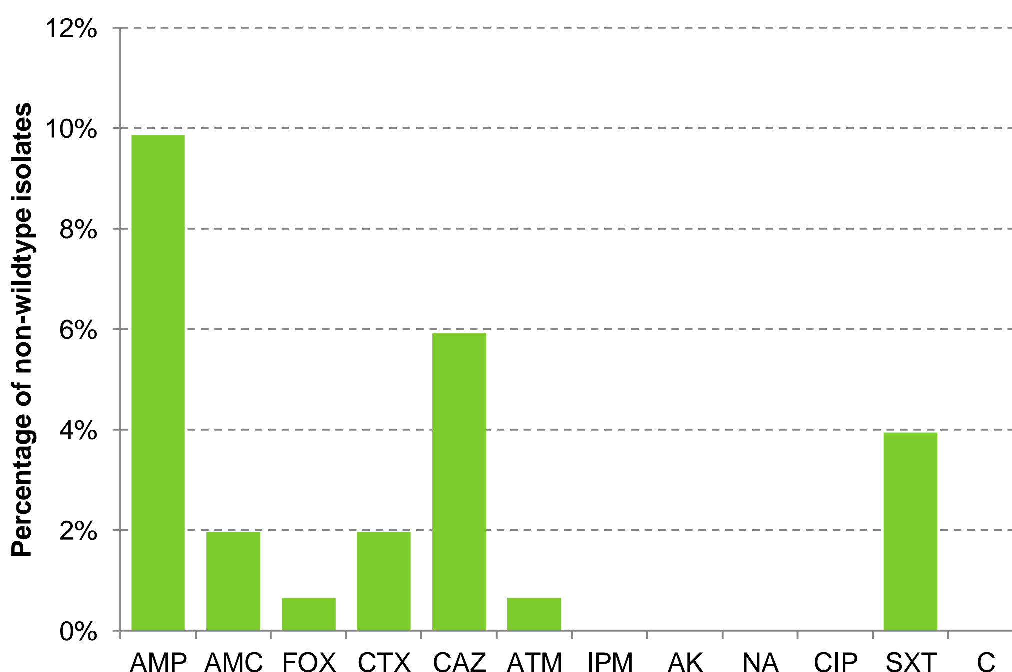

Figure 16: Percentage of non-wild type *E. coli* isolates according to epidemiological cut-offs, considering the total number of isolates. AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; C = chloramphenicol.

Focusing on the values obtained for each of the ungulates species (Figure 17), only one *E. coli* from roe deer showed a non-wild type phenotype for the antibiotic aztreonam. The percentage of acquired resistance for ampicillin was higher in the wild boar isolates (22%) than in the red deer's (3%). The same trend was observed for ceftazidime and co-trimoxazole. Non-wild type phenotype for cefoxitin was detected exclusively in one strain, isolated from a red deer from Idanha-a-Nova region. Interestingly, this strain was susceptible to all the other β-lactams. A study in an urban area in Barcelona with wild boars had reported similar results, having characterized the studied population as non-wild type to tetracycline, streptomycin, trimethoprim,

sulfamethoxazole and ampicillin (Nora Navarro-Gonzalez *et al.*, 2013). In another study involving wild boars inhabiting a National Game Reserve (Spain) it was observed that the higher percentage of non-wild type *E. coli* was obtained for tetracycline, followed by ampicillin (N Navarro-Gonzalez *et al.*, 2013).

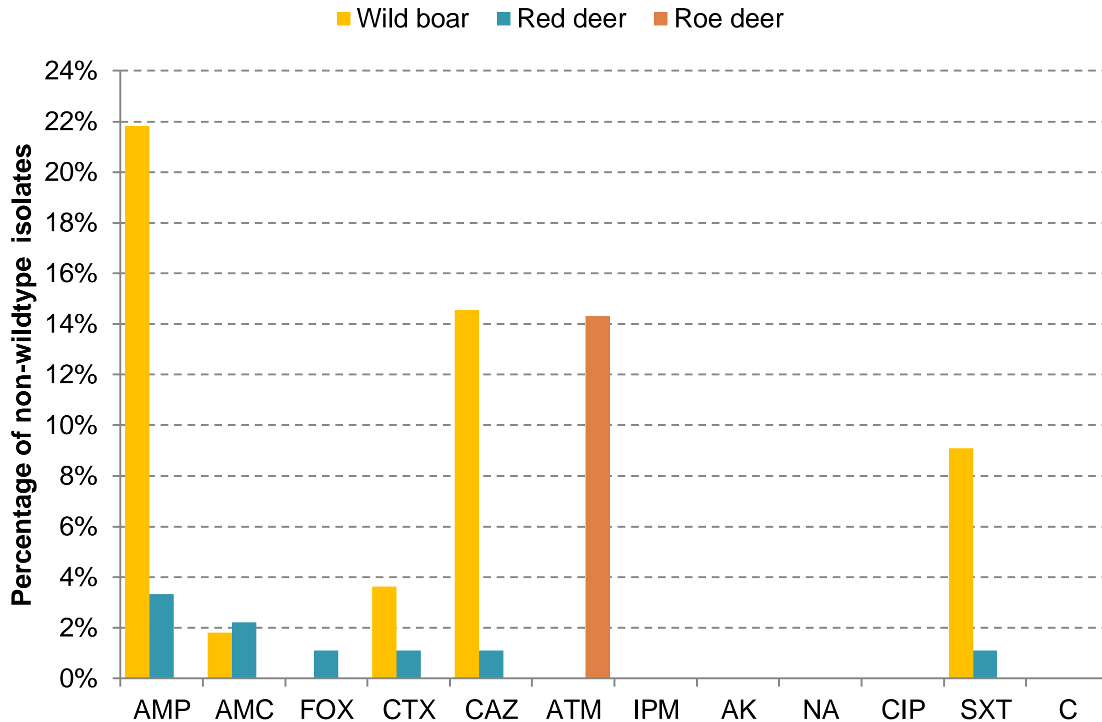

Figure 17: Percentage of non-wild type *E. coli* isolates according to epidemiological cut-offs, considering the isolates collected from wild boar, red deer and roe deer independently. AMP = ampicillin; AMC = amoxicillin/clavulanic acid; FOX = cefoxitin; CTX = cefotaxime; CAZ = ceftazidime; ATM = aztreonam; IPM = imipenem; AK = amikacin; NA = nalidixic acid; CIP = ciprofloxacin; SXT = co-trimoxazole; C = chloramphenicol.

ECOFFs can be employed in populations of bacteria from distinct sources if the wild type distributions are independent of the host species (Santos *et al.*, 2013). The distribution of IZDs observed for the *E. coli* strains recovered from wild ungulates was analyzed for ampicillin, co-trimoxazole, cefotaxime and ceftazidime (Figure 18). Also, the data provided by the EUCAST for the same species and antibiotics was included for comparison. It was found that the distribution of IZDs of the strains from wild ungulates and the EUCAST strains were highly similar for ampicillin and co-trimoxazole antibiotics. However, the same was not observed for cefotaxime and ceftazidime. For these two antibiotics, the IZDs obtained in the present study follow the typical Gaussian distribution. However, their medians were about 2 to 4 mm smaller than the IZDs of EUCAST for cefotaxime and ceftazidime, respectively. These results can be due to the difference in the

number of isolates considered, although this factor was not critical for the analysis of the other two antibiotics. On the other hand, the results can also indicate that the distribution of IZDs is slightly different and in this situation the use of ECOFFs can lead to an overestimation of non-wild type phenotypes. This issue should be further investigated through the MICs distribution analysis of the 152 isolates presented in this study for both cefotaxime and ceftazidime.

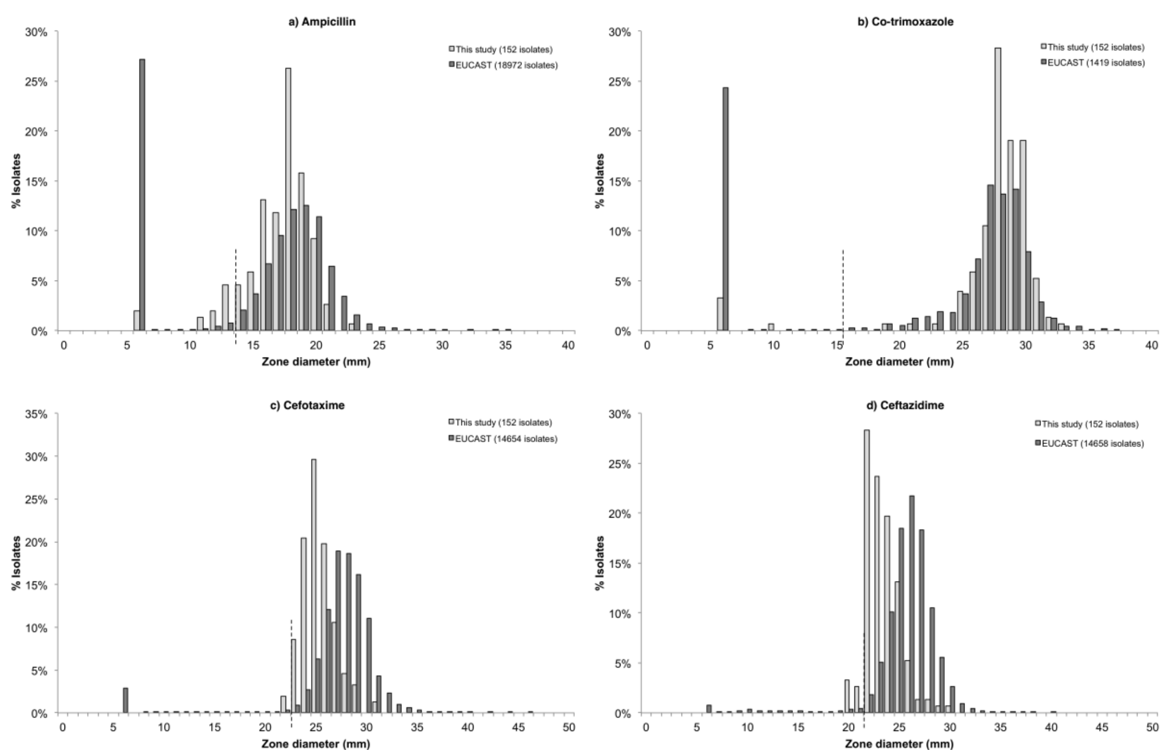

Figure 18: Histograms of distribution of the inhibition zone diameters obtained for the *E. coli* isolates object of the present study and those provided by EUCAST to ampicillin, co-trimoxazole, cefotaxime and ceftazidime. The dashed line indicates the epidemiological cut-off value defined for the wild type population by EUCAST.

As abovementioned, the ECOFFs for tetracycline and streptomycin are not defined for *E. coli* by EUCAST, considering disk diffusion testing. Thus, the distribution of the IZDs obtained in this study for these two antibiotics were analyzed (Figure 19). According to this analysis, the ECOFFs of 13 mm and 19 mm could be proposed for streptomycin and tetracycline, respectively. Considering these values, the percentage of non-wild type isolates for tetracycline would be 9% and for streptomycin 5%. Yet, this information will not be considered to define resistance profiles in the following sections, since it is based on non-validated cut-offs. Thus, it would be also important to obtain the tetracycline and streptomycin ECOFFs for *E. coli* based on disk diffusion testing, as it was already performed for the broth dilution testing.

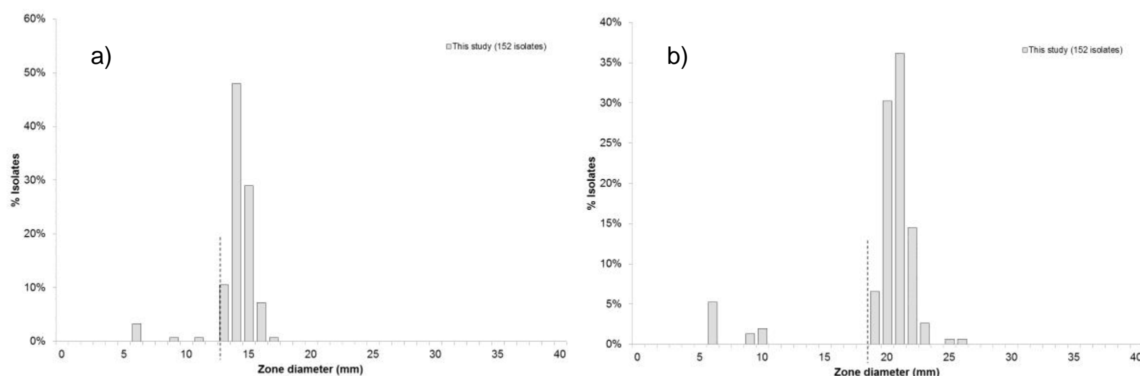

Figure 19: Histogram of the IZDs obtained for the *E. coli* isolates object of the present study to a) streptomycin and b) tetracycline. The dashed line indicates our proposal to ECOFF for the wild type population.

3.3.3. Resistance phenotypes

According to the clinical breakpoints values, a multiresistance phenotype was identified for 5 isolates (3.3%), which showed resistance to ampicillin, streptomycin, co-trimoxazole and tetracycline (Table 6). Co-resistance of ampicillin with amoxicillin/clavulanic acid (n=2) or with co-trimoxazole (n=1) was also observed. Using the epidemiological cut-offs the number of isolates possessing a co-resistance phenotype was the same (n= 10; Table 7). The multiresistant (n=5; AMP^R-SXT^R-S^R-TE^R) and the AMP^R-SXT^R (n=1) isolates defined according to the clinical breakpoints matched with the 6 isolates identified with AMP^R-SXT^R phenotype defined by the ECOFFs. This was not surprising because streptomycin and tetracycline were not herein considered for the analysis with epidemiological cut-offs. Five of these isolates were collected from wild boar and one from red deer samples, all inhabiting Lousã area. Considering the 5 isolates from wild boar, despite the common resistance phenotype, only 2 isolates (J1-6 and J3-12) originated a similar banding profile in BOX-PCR analysis, suggesting their clonal relationship. The co-resistance to penicillins and cephalosporins was identified only in one isolate and according to ECOFFs. The most frequent detected phenotypes with clinical breakpoints were AMP^R (n=7) and TE^R (n=6); moreover, the most non-wild type phenotypes detected were CAZ^R (n=7), AMP^R (n=6) and AMP^R-SXT^R (n=6).

Independently of the system used for the interpretation of IZDs, the multiresistant isolates herein identified were mainly recovered from wild boar samples. The phenotype of these isolates was characterized by resistance to ampicillin, streptomycin, tetracycline and co-trimoxazole. Interestingly, the AMP^R-S^R-T^R has been reported as the most abundant non-ESBL resistance phenotype among *E. coli* of wildlife origin (Guenther *et al.*, 2011). In fact, herein, the co-resistance of penicillins and cephalosporins was observed

only in one strain, suggesting a very low prevalence of ESBL-producers. In Portugal, the percentages of ESBLs detected in wildlife ranged from 10% (in wild boars) to 30% (in seagulls) (Poeta *et al.*, 2009; Simões *et al.*, 2010).

Table 6: Resistance phenotypes detected among the 152 *E. coli* isolates obtained from faecal samples of wild ungulates in Portugal, according to clinical breakpoints.

Resistance phenotype	Total of isolates	Isolates	
		Wild boar	Red deer
AMP-S-SXT-TE	5	J1-6; J2-1; J3-12; J3-5	V2-7
AMP-AMC	2	J8-8	V19-1
AMP-SXT	1	J2-4	-
S-TE	2	-	V35-1; V7-3
AMP	7	J1-1; J2-5; J7-2; J4-1; J14-10; J20-6	V23-7
AMC	1	-	V28-3
FOX	1	-	V26-7
TE	6	J3-10; J4-2	V5v-4; V3-5; V33-2; V34-8

Table 7: Resistance phenotypes detected among the 152 *E. coli* isolates obtained from faecal samples of wild ungulates in Portugal, according to epidemiological cut-offs.

Non-wild type phenotype	Number of isolates	Isolates		
		Wild boar	Red deer	Roe deer
AMP-AMC	2	J8-8	V19-1	-
AMP-CAZ	1	J7-2	-	-
AMP-SXT	6	J1-6; J2-1; J2-4; J3-5; J3-12	V2-7	-
CAZ-CTX	1	J6-1	-	-
AMP	6	J1-1; J2-5; J4-1; J14-10; J20-6	V23-7	-
AMC	1	-	V28-3	-
ATM	1	-	-	V/C-3
CAZ	7	J4-4; J7-5; J9-8; J16-3; J13-8; J13-9	V23-8	-
CTX	2	12-7	V1-10	-
FOX	1	-	V26-7	-

3.4. Screening of shiga toxin-producing *Escherichia coli* (STEC)

3.4.1. Samples

The 67 faecal samples were screened for the presence of STEC by amplification of shiga-toxin genes *stx1* and *stx2*. STEC strains are generally divided in O157:H7 and non-O157 serotype. Thus, the same samples were also investigated specifically for the presence of the *E. coli* O157:H7 serotype using the *rfbE* gene as a marker. The results of real-time PCR showed that 19.4% of the samples (n=13) were positive for at least one of the shiga-toxin genes (Table 8). Among these, a roe deer's sample was positive for both *stx* variants under study. Regarding the *rfbE* gene, it was successfully amplified only in one sample (1.5%), from wild boar (Table 8), indicating the presence of *E. coli* O157:H7 in this animal. Considering each species of the wild ungulates under study, STEC-associated genes were identified in 4.5% of the wild boar samples, 13.4% of the red deer and 1.5% of the roe deer. In order to confirm the results, PCR reactions targeting *stx1* and *stx2* individually were performed for the thirteen positive samples. The results obtained were in agreement with those obtained with multiplex PCR. Thus, it was determined that 31% of the positive samples possess the *stx1* gene, whereas 77% have the *stx2* variant (Figure 20).

Table 8: List of positive faecal samples for the shiga-toxin genes amplified in this study by real-time PCR.

Sample ID	Source	Geographical area	Gene amplified
V2		Lousã	*stx2*
V8		Lousã	*stx2*
V18		Lousã	*stx2*
V23		Idanha-a-Nova	*stx2*
V25	Red deer	Idanha-a-Nova	*stx2*
V27		Idanha-a-Nova	*stx2*
V32		Lousã	*stx2*
V38		Lousã	*stx1*
V39		Lousã	*stx1*
J5		Lousã	*stx2*
J7	Wild boar	Lousã	*stx1*
J21		Idanha-a-Nova	*stx2; rfbE*
V/C	Roe deer	Montesinho	*stx1* and *stx2*

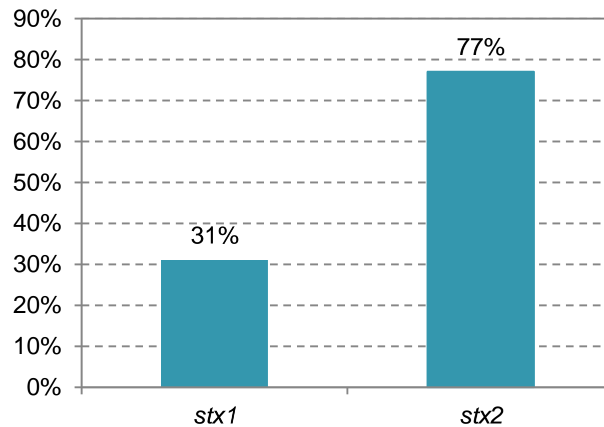

Figure 20: Percentage of *stx* variants detected in the 13 positive faecal samples.

In Spain, an investigation carried out with sheep showed that non-O157 STEC strains were present in 35% of the animals, whereas O157:H7 STEC were recovered only from 1% (Rey *et al.*, 2003). Other study involving a wild boar population of Spain detected STEC in 8% of the sampled animals, in which 3,3% were *E. coli* O157:H7 and 5,2% were non-O157 STEC strains (Sánchez *et al.*, 2010). Consequently, despite some differences, the occurrence of non-O157 STEC isolates is generally higher in animals than the occurrence of O157:H7 strains. Therefore, our results are in agreement with these studies, since the percentage of wild ungulates carrying non-O157 STEC strains should be higher than those possessing O157:H7 STEC strains.

3.4.2. *E. coli* isolates

The *E. coli* strains (n=31) isolated from the 13 STEC-positive samples were screened for the presence of *rfbE*, *stx1* and *stx2* genes. This approach allowed to detect 3 isolates possessing the *stx1* (n=1) and the *stx2* (n=2) genes, whereas none of them was positive for the *rfbE* gene (Table 9). These 3 isolates were recovered from red and roe deer and are non-O157 STEC. After the sequencing reaction, it was possible to determine the subtype of each of the *stx* genes amplified (Table 9). Some studies suggest that Stx2 toxin alone can cause symptoms of hemolytic-uremic syndrome (HUS), whereas the administration of the same dose of Stx1 does not cause such symptoms (Fuller *et al.*, 2011). Particularly, the subtypes *stx2a*, *stx2c* and *stx2d* have been linked with severe illness in humans (Feng *et al.*, 2011). In this study, only one of these variants (*stx2d*) was identified in the strain V23-9, isolated from red deer inhabiting Idanha-a-Nova.

Table 9: List of *E. coli* isolates positive for *stx* genes.

Sample ID	Isolate	Source	Geographical area	Gene amplified
V23	V23-9	Red deer	Idanha-a-Nova	*stx2d*
V32	V32-8		Lousã	*stx2g, ehxA*
V/C	V/C-3	Roe deer	Montesinho	*stx1c*

It is known that STEC strains normally encode other virulence factors in addition to *stx*. One example is the outer membrane protein intimin, encoded by the *eae* gene. This gene is part of a pathogenicity island called locus of enterocyte effacement (LEE). The LEE possesses the genetic determinants responsible for the ability of some *E. coli* isolates to cause lesions on intestinal epithelial cells (Blanco *et al.*, 2005). Other important virulence factors are the hemolysins, such as the encoded by the plasmid-carried *ehxA* gene (Lorenz *et al.*, 2013). This gene is frequently associated with diarrheal disease in humans. Thus, the presence of *eae* and *ehxA* in the 3 STEC isolates identified in this study was investigated. The strain V32-8, possessing the *stx2g* gene, was also positive for *ehxA* and none of the isolates amplified the intimin gene. In fact, in wildlife, the occurrence of enterohemolysin genes in STEC strains is usually higher than *eae* (Sánchez *et al.*, 2010).

4. Conclusions and Future Perspectives

Wildlife is being recognized as a reservoir of pathogenic bacteria such as STEC and *Salmonella* spp. Moreover, the number of studies reporting the occurrence of AMR in wildlife is increasing, including resistance towards antibiotics commonly used in human therapeutics (Miko *et al.*, 2009; Cristóbal-Azkarate *et al.*, 2014). Thus, in the present study, three Portuguese wild ungulates were studied to evaluate the levels of AMR present in their *E. coli* isolates and also to understand their potential as carriers of potential harmful bacteria to humans. To achieve this, a total of 67 faecal samples from red deer, wild boar and roe deer inhabiting three distinct geographical areas were collected and analyzed. 152 *E. coli* isolates were submitted to antimicrobial susceptibility testing and it was found that highest levels of resistance were obtained for β-lactams (mainly ampicillin) and tetracyclines. On the other hand, resistance to chloramphenicol and to the synthetic antibiotics (quinolones and fluoroquinolones) was not identified. The percentage of non-wild type phenotypes detected using ECOFFs was equal to the percentage of resistant isolates established with clinical breakpoints for ampicillin, amoxicillin/clavulanic acid, cefoxitin and co-trimoxazole. For ceftazidime and azetreonam, they corresponded exactly to the percentage of intermediate resistant isolates identified with clinical breakpoints. The analysis with ECOFFs allowed the identification of a non-wild type phenotype for cefotaxime, whereas the population was entirely susceptible to this antibiotic from the clinical point of view. Thus, it was possible to identify isolates with non-wild type phenotypes towards cephalosporins of third generation. *E. coli* strains from wild boar showed the highest values of resistance, followed by red deer. No resistant isolates were identified from roe deer. It was found that 3.3% of the bacteria isolates studied are multiresistant, possessing the AMP^R-S^R-SXT^R-TE^R phenotype. All these bacteria were collected from animals living in Lousã. None of the isolates presented a resistance phenotype indicating the presence of ESBLs, which hydrolyze β-lactams, broad-spectrum cephalosporins and monobactams. Hereafter, it will be important to characterize the resistance genes present in the isolates that reveal a resistance phenotype. Moreover, it would be interesting to study the presence of mobile genetic elements, such as integrons, frequently associated with the recruitment of AMR genes.

Considering the presence of bacteria potentially pathogenic to humans, a low prevalence of *Salmonella* spp. was observed in the present study. Only one isolate was recovered, with susceptibility to all the antibiotics tested. The isolate was not characterized, so far, in terms of subspecies and serovar, but that will be performed in the future. In forthcoming studies, it would be also interesting to adopt molecular-based techniques to complement the cultivation-dependent methods used for the detection of

these bacteria. The use of PCR for the identification of STEC allowed to perceive that 19.4% (n=13) of the wild ungulates included in this study are potential carriers of STEC, including the enterohemorrhagic serotype O157:H7. The results obtained suggest that *stx2* genes are more abundant than *stx1* in the positive samples. Also, the *E. coli* strains previously isolated from STEC-positive samples were examined for the presence of shiga-toxin genes. With this approach it was possible to detect 3 STEC strains, all from different samples. Thus, none of the isolates from the remaining 10 samples were STEC. However, it should be considered that the selection of *E. coli* colonies was not performed from enriched cultures. These cultures were preserved in order to evaluate the presence of STEC using rainbow agar and chromagar STEC. Nevertheless, it is confirmed that at least 4.5% of the wild ungulates of this study are STEC reservoirs, containing the *stx1c*, *stx2d* and *stx2g* variants of shiga-toxin genes. Moreover, one of these isolates also bear the enterohemolysin gene *ehxA*. None of the isolates were positive for *eae* gene. To the best of our knowledge, this is the first report of STEC occurrence in Portuguese red and roe deer animals. These isolates should be further characterized mainly regarding their serotype and the production of toxins.

The study herein presented, although at an early stage, reveals that surveillance studies are needed and should be continued in the future to follow up the prevalence of AMR and human pathogens in wild animals. Moreover, data regarding the emergence and dissemination of new or already known AMR genes in these different ecosystems should be collected. The three geographical areas chosen for this study are characterized by distinct anthropogenic influences. Therefore, it would be important to collect and analyse approximately the same number of *E. coli* isolates from these three regions. This will allow to understand if a correlation exists between the proximity to humans and AMR prevalence in the wild animals, as suggested by several studies. Nevertheless, our results show that common Portuguese wild ungulates can be reservoirs of antibiotic resistant bacteria and may act as carriers for their transmission. Furthermore, to prevent the occurrence of zoonoses, it is important to identify which populations and animals can be the primary source of infection.

In summary, the following major points require further investigation:

i. Characterization of the antimicrobial resistance genes;
ii. Characterization of the *Salmonella* spp. isolate serotype;
iii. Investigation of the presence of STEC using agar-based techniques and the cultures after enrichment;

iv. Characterization of the STEC isolates collected in this study regarding their serotype, production of toxin and presence of other virulence genes, such as *saa* (STEC autoagglutinating adhesin);
v. Determination and analysis of the *E. coli* phylogenetic groups.

Bibliography

(DGAV) Direcção-geral de alimentação Veterinária (2011) *Relatório nacional de monitorização de consumo de antimicrobianos*. Portugal.

Acevedo P, Quirós-Fernández F, Casal J & Vicente J (2014) Spatial distribution of wild boar population abundance: Basic information for spatial epidemiology and wildlife management. *Ecol Indic* **36**: 594–600.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J & Handelsman J (2010) Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol* **8**: 251–259.

Allen SE, Boerlin P, Janecko N, Lumsden JS, Barker IK, Pearl DL, Reid-Smith RJ & Jardine C (2011) Antimicrobial resistance in generic Escherichia coli isolates from wild small mammals living in swine farm, residential, landfill, and natural environments in southern Ontario, Canada. *Appl Environ Microbiol* **77**: 882–888.

Alves J, Alves da Silva A, Soares AMVM & Fonseca C (2014) Spatial and temporal habitat use and selection by red deer: The use of direct and indirect methods. *Mamm Biol - Zeitschrift für Säugetierkd* **79**: 338–348.

Alves MS, Pereira A, Araújo SM, Castro BB, Correia ACM & Henriques I (2014) Seawater is a reservoir of multi-resistant Escherichia coli, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. *Front Microbiol* **5**: 426.

Amaro A (2011) Susceptibilidade a antibióticos em isolados bacterianos do ambiente em lares de 3ª idade. Instituto Politécnico de Bragança.

Atwater RM (1950) The Merck Manual of Diagnosis and Therapy. *Am J Public Heal Nations Heal* **40**: 1454–1454.

Bai J, Shi X & Nagaraja TG (2010) A multiplex PCR procedure for the detection of six major virulence genes in Escherichia coli O157:H7. *J Microbiol Methods* **82**: 85–89.

Bertrand R & Roig B (2007) Evaluation of enrichment-free PCR-based detection on the rfbE gene of Escherichia coli O157--application to municipal wastewater. *Water Res* **41**: 1280–1286.

Blanco M, Schumacher S, Tasara T, Zweifel C, Blanco JE, Dahbi G, Blanco J & Stephan R (2005) Serotypes, intimin variants and other virulence factors of eae positive Escherichia coli strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (eae-eta2). *BMC Microbiol* **5**: 23.

Botti V, Navillod FV, Domenis L, Orusa R, Pepe E, Robetto S & Guidetti C (2010) Salmonella spp. and antibiotic-resistant strains in wild mammals and birds in north-western Italy from 2002 to 2010. 195–202.

Bronzwaer SLAM, Goettsch W, Olsson-Liljequist B, Wale MCJ, Vatopoulos AC & Sprenger MJ. (1999) European Antimicrobial Resistance Surveillance System

(EARSS): objectives and organisation. *Euro Surveill Bull Eur sur les Mal Transm = Eur Commun Dis Bull* **4**: 41–44.

Brown D (2011) EUCAST definitions. *European Congress of Clinical Microbiology and Infectious Diseases*. ESCMID, Milan.

Caleja C, Toro M De, Gonçalves A, *et al.* (2011) Antimicrobial resistance and class I integrons in Salmonella enterica isolates from wild boars and Bísaro pigs. 19–24.

Caprioli A, Donelli G, Falbo V, Passi C, Pagano A & Mantovani A (1991) Antimicrobial resistance and production of toxins in Escherichia coli strains from wild ruminants and the alpine marmot. *J Wildl Dis* **27**: 324–327.

CDC (2013) *Antibiotic resistance threats in the United States, 2013*.

Cepo Verde Turismo Rural L Turismo de Natureza no parque natural de Montesinho. http://www.montesinho.com/?menu=oparque (Accessed November 4, 2014).

Chiari M, Zanoni M, Tagliabue S, Lavazza A & Alborali LG (2013) Salmonella serotypes in wild boars (Sus scrofa) hunted in northern Italy. *Acta Vet Scand* **55**: 42.

Clermont O, Christenson JK, Denamur E & Gordon DM (2013) The Clermont Escherichia coli phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep* **5**: 58–65.

Cooley MB, Jay-Russell M, Atwill ER, *et al.* (2013) Development of a robust method for isolation of shiga toxin-positive Escherichia coli (STEC) from fecal, plant, soil and water samples from a leafy greens production region in California. *PLoS One* **8**: e65716.

Costa D, Poeta P, Sáenz Y, Vinué L, Coelho AC, Matos M, Rojo-Bezares B, Rodrigues J & Torres C (2008) Mechanisms of antibiotic resistance in Escherichia coli isolates recovered from wild animals. *Microb Drug Resist* **14**: 71–77.

Cristóbal-Azkarate J, Dunn JC, Day JMW & Amábile-Cuevas CF (2014) Resistance to antibiotics of clinical relevance in the fecal microbiota of Mexican wildlife. *PLoS One* **9**: e107719.

Dantas G & Sommer MO a. (2014) How to Fight Back Against Antibiotic Resistance. *Am Sci* **102**: 42.

Davies J & Davies D (2010) Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**: 417–433.

EFSA & ECDC (2014) *The European Union Summary Report on antimicrobial resistance in zoonotic Antimicrobial resistance in zoonotic and indicator bacteria from humans , animals and food in the European Union in 2012*.

EUCAST (2013) Antimicrobial susceptibility testing EUCAST disk diffusion method.

European Centre for Disease Prevention and Control. (2014) *Antimicrobial resistance surveillance in Europe 2013. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net).* Stockholm: ECDC.

European Food Safety Authority (2014) EFSA explains zoonotic diseases: Food-borne zoonotic diseases.

European Medicines Agency (2013) Sales of veterinary antimicrobial agents in 25 EU / EEA countries in Third ESVAC report. *Eur Med Agency* 57.

Feng PCH, Jinneman K, Scheutz F & Monday SR (2011) Specificity of PCR and serological assays in the detection of Escherichia coli Shiga toxin subtypes. *Appl Environ Microbiol* **77**: 6699–6702.

Ferreira E, Souto L, Soares AMVM & Fonseca C (2009) Genetic structure of the wild boar population in Portugal: Evidence of a recent bottleneck. *Mamm Biol - Zeitschrift für Säugetierkd* **74**: 274–285.

Fonseca C, Alves J, Silva AA da & Soares AMVM (2009) Gestão de Habitat para Ungulados em Ecossistema Montanhoso: o caso da Serra da Lousã. 8.

Fuller CA, Pellino CA, Flagler MJ, Strasser JE & Weiss AA (2011) Shiga toxin subtypes display dramatic differences in potency. *Infect Immun* **79**: 1329–1337.

Gansheroff LJ & O’Brien AD (2000) Escherichia coli O157:H7 in beef cattle presented for slaughter in the U.S.: Higher prevalence rates than previously estimated. *Proc Natl Acad Sci* **97**: 2959–2961.

Gilliver MA, Bennett M, Begon M, Hazel SM & Hart CA (1999) Antibiotic resistance found in wild rodents. *Nature* **401**: 233–234.

Gonçalves A, Igrejas G, Radhouani H, Estepa V, Alcaide E, Zorrilla I, Serra R, Torres C & Poeta P (2012) Detection of extended-spectrum beta-lactamase-producing Escherichia coli isolates in faecal samples of Iberian lynx. *Lett Appl Microbiol* **54**: 73–77.

Gonçalves A, Igrejas G, Radhouani H, *et al.* (2013) Antimicrobial resistance in faecal enterococci and Escherichia coli isolates recovered from Iberian wolf. *Lett Appl Microbiol* **56**: 268–274.

Gonçalves A, Igrejas G, Radhouani H, *et al.* (2013) Detection of antibiotic resistant enterococci and Escherichia coli in free range Iberian Lynx (Lynx pardinus). *Sci Total Environ* **456-457**: 115–119.

Gould LH, Mody RK, Ong KL, *et al.* (2013) Increased recognition of non-O157 Shiga toxin-producing Escherichia coli infections in the United States during 2000-2010: epidemiologic features and comparison with E. coli O157 infections. *Foodborne Pathog Dis* **10**: 453–460.

Guenther S, Ewers C & Wieler LH (2011) Extended-Spectrum Beta-Lactamases Producing E. coli in Wildlife, yet Another Form of Environmental Pollution? *Front Microbiol* **2**: 246.

Hussein HS (2007) Prevalence and pathogenicity of Shiga toxin-producing Escherichia coli in beef cattle and their products. *J Anim Sci* **85**: E63–E72.

International Organization for Standardization (2007) Annex D: Detection of Salmonella spp. in animal faeces and in environmental samples from the primary production stage. **2007**.

Jehl F, Chomarat M, Tankovic J & Gérard A (2012) *De L'antibiogramme à la prescription*. ÉDITIONS BIOMÉRIEUX.

Kahlmeter G (2011) Common epidemiological cutoff values (ECOFFs) for surveillance of resistance – is it feasible? *International Meeting on Emerging Diseases and Surveilance*. International Society for Infectious Diseases, Wien.

Lewis K (2013) Platforms for antibiotic discovery. *Nat Rev Drug Discov* **12**: 371–387.

Literak I, Dolejska M, Radimersky T, Klimes J, Friedman M, Aarestrup FM, Hasman H & Cizek A (2010) Antimicrobial-resistant faecal Escherichia coli in wild mammals in central Europe: multiresistant Escherichia coli producing extended-spectrum beta-lactamases in wild boars. *J Appl Microbiol* **108**: 1702–1711.

Lorenz SC, Son I, Maounounen-Laasri A, Lin A, Fischer M & Kase JA (2013) Prevalence of hemolysin genes and comparison of ehxA subtype patterns in Shiga toxin-producing Escherichia coli (STEC) and non-STEC strains from clinical, food, and animal sources. *Appl Environ Microbiol* **79**: 6301–6311.

Lupo A, Coyne S & Berendonk TU (2012) Origin and evolution of antibiotic resistance: The common mechanisms of emergence and spread in water bodies. *Front Microbiol* **3**.

Lupski JR & Weinstock GM (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* **174**: 4525–4529.

Marathe SA, Chowdhury R, Bhattacharya R, Nagarajan AG & Chakravortty D (2012) Direct detection of Salmonella without pre-enrichment in milk, ice-cream and fruit juice by PCR against hilA gene. *Food Control* **23**: 559–563.

Martínez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. *Science* **321**: 365–367.

McDaniels AE, Rice EW, Reyes AL, Johnson CH, Haugland RA & Stelma GN (1996) Confirmational identification of Escherichia coli, a comparison of genotypic and phenotypic assays for glutamate decarboxylase and beta-D-glucuronidase. *Appl Environ Microbiol* **62**: 3350–3354.

Miko A, Pries K, Haby S, Steege K, Albrecht N, Krause G & Beutin L (2009) Assessment of Shiga toxin-producing Escherichia coli isolates from wildlife meat as potential pathogens for humans. *Appl Environ Microbiol* **75**: 6462–6470.

Navarro-Gonzalez N, Mentaberre G, Porrero CM, Serrano E, Mateos A, López-Martín JM, Lavín S & Domínguez L (2012) Effect of cattle on Salmonella carriage, diversity and antimicrobial resistance in free-ranging wild boar (Sus scrofa) in northeastern Spain. *PLoS One* **7**: e51614.

Navarro-Gonzalez N, Porrero MC, Mentaberre G, Serrano E, Mateos A, Domínguez L & Lavín S (2013) Antimicrobial resistance in indicator Escherichia coli isolates from free-ranging livestock and sympatric wild ungulates in a natural environment (Northeastern Spain). *Appl Environ Microbiol* **79**: 6184–6186.

Navarro-Gonzalez N, Casas-Díaz E, Porrero CM, Mateos A, Domínguez L, Lavín S & Serrano E (2013) Food-borne zoonotic pathogens and antimicrobial resistance of indicator bacteria in urban wild boars in Barcelona, Spain. *Vet Microbiol* **167**: 686–689.

Núcleo Executivo de Idanha-a-Nova (2005) Diagnóstico Social do Município de Idanha-a-Nova.

Osterblad M, Norrdahl K, Korpimäki E & Huovinen P (2001) Antibiotic resistance. How wild are wild mammals? *Nature* **409**: 37–38.

Pacheco AR & Sperandio V (2012) Shiga toxin in enterohemorrhagic E.coli: regulation and novel anti-virulence strategies. *Front Cell Infect Microbiol* **2**: 81.

Pathmanathan SG (2003) Simple and rapid detection of Salmonella strains by direct PCR amplification of the hilA gene. *J Med Microbiol* **52**: 773–776.

Persson S, Olsen KEP, Ethelberg S & Scheutz F (2007) Subtyping method for Escherichia coli shiga toxin (verocytotoxin) 2 variants and correlations to clinical manifestations. *J Clin Microbiol* **45**: 2020–2024.

Pesapane R, Ponder M & Alexander KA (2013) Tracking pathogen transmission at the human-wildlife interface: banded mongoose and Escherichia coli. *Ecohealth* **10**: 115–128.

Philpott D & Ebel F (2002) *E. coli*. Humana Press, New Jersey.

Piérard D, Van Damme L, Moriau L, Stevens D & Lauwers S (1997) Virulence factors of verocytotoxin-producing Escherichia coli isolated from raw meats. *Appl Environ Microbiol* **63**: 4585–4587.

Pitout JDD (2012) Extraintestinal Pathogenic Escherichia coli: A Combination of Virulence with Antibiotic Resistance. *Front Microbiol* **3**: 9.

Poeta P, Radhouani H, Pinto L, *et al.* (2009) Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing Escherichia coli of different phylogenetic groups. *J Basic Microbiol* **49**: 584–588.

Quinteira S (1999) Resistências aos antibióticos B-lactâmicos em Enterobacteriaceae isoladas de águas de consumo. Faculdade de Farmácia da Universidade do Porto.

Rademaker JLW & De Bruijn. FJ (1997) Characterization and classification of microbes by Rep-PCR genomic fingerprinting and computer-assisted pattern analysis. *DNA Markers: Protocols, Applications, and Overviews*, pp. 151–171.

Radhouani H, Igrejas G, Gonçalves A, Pacheco R, Monteiro R, Sargo R, Brito F, Torres C & Poeta P (2013) Antimicrobial resistance and virulence genes in Escherichia coli and enterococci from red foxes (Vulpes vulpes). *Anaerobe* **23**: 82–86.

Radhouani H, Silva N, Poeta P, Torres C, Correia S & Igrejas G (2014) Potential impact of antimicrobial resistance in wildlife, environment and human health. *Front Microbiol* **5**: 23.

Rey J, Blanco JE, Blanco M, *et al.* (2003) Serotypes, phage types and virulence genes of Shiga-producing Escherichia coli isolated from sheep in Spain. *Vet Microbiol* **94**: 47–56.

Rwego IB, Isabirye-Basuta G, Gillespie TR & Goldberg TL (2008) Gastrointestinal bacterial transmission among humans, mountain gorillas, and livestock in Bwindi Impenetrable National Park, Uganda. *Conserv Biol* **22**: 1600–1607.

Sánchez S, Martínez R, García A, *et al.* (2010) Detection and characterisation of O157:H7 and non-O157 Shiga toxin-producing Escherichia coli in wild boars. *Vet Microbiol* **143**: 420–423.

Santos J, Carvalho J, Torres R, Gortázar C & Fonseca C (2011) Red deer in northeastern Portugal: monitoring trends in a transboundary population. *XXXth IUGB Congress (International Union of Game Biologists) and Perdix XIII*. Barcelona, Spain.

Santos T, Silva N, Igrejas G, *et al.* (2013) Dissemination of antibiotic resistant Enterococcus spp. and Escherichia coli from wild birds of Azores Archipelago. *Anaerobe* **24**: 25–31.

Sayah RS, Kaneene JB, Johnson Y & Miller R (2005) Patterns of antimicrobial resistance observed in Escherichia coli isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. *Appl Environ Microbiol* **71**: 1394–1404.

Shakil S, Khan R, Zarrilli R & Khan AU (2008) Aminoglycosides versus bacteria--a description of the action, resistance mechanism, and nosocomial battleground. *J Biomed Sci* **15**: 5–14.

Sharma VK (2006) Real-time reverse transcription-multiplex PCR for simultaneous and specific detection of rfbE and eae genes of Escherichia coli O157:H7. *Mol Cell Probes* **20**: 298–306.

Silva N, Igrejas G, Gonçalves A & Poeta P (2011) Commensal gut bacteria: distribution of Enterococcus species and prevalence of Escherichia coli phylogenetic groups in animals and humans in Portugal. *Ann Microbiol* **62**: 449–459.

Simões RR, Poirel L, Da Costa PM & Nordmann P (2010) Seagulls and beaches as reservoirs for multidrug-resistant Escherichia coli. *Emerg Infect Dis* **16**: 110–112.

Sjölund M, Bonnedahl J, Hernandez J, Bengtsson S, Cederbrant G, Pinhassi J, Kahlmeter G & Olsen B (2008) Dissemination of multidrug-resistant bacteria into the Arctic. *Emerg Infect Dis* **14**: 70–72.

Sjölund M, Bengtsson S, Bonnedahl J, Hernandez J, Olsen B & Kahlmeter G (2009) Antimicrobial susceptibility in Escherichia coli of human and avian origin--a comparison of wild-type distributions. *Clin Microbiol Infect* **15**: 461–465.

Smith S, Wang J, Fanning S & McMahon BJ (2014) Antimicrobial resistant bacteria in wild mammals and birds: a coincidence or cause for concern? *Ir Vet J* **67**: 8.

Son I, Binet R, Maounounen-Laasri A, Lin A, Hammack TS & Kase J a (2014) Detection of five Shiga toxin-producing Escherichia coli genes with multiplex PCR. *Food Microbiol* **40**: 31–40.

Stefani S (2012) Analyzing possible intersections in the resistome among human, animal, and environment matrices. *Front Microbiol* **3**: 418.

Torres R, Dias D, Caetano T & Mendo S (2014) Antimicrobial resistance in wild boar (Sus scrofa) in Portugal. *10th International symposium on wild boar and other suids*. ERICo, Velenje, Slovenia.

Torres RT, Santos J, Linnell JDC, Virgós E & Fonseca C (2011) Factors affecting roe deer occurrence in a Mediterranean landscape, Northeastern Portugal. *Mamm Biol - Zeitschrift für Säugetierkd* **76**: 491–497.

Torres RT, Virgós E, Panzacchi M, Linnell JDC & Fonseca C (2012) Life at the edge: Roe deer occurrence at the opposite ends of their geographical distribution, Norway and Portugal. *Mamm Biol - Zeitschrift für Säugetierkd* **77**: 140–146.

Valente AM, Fonseca C, Marques T a, Santos JP, Rodrigues R & Torres RT (2014) Living on the edge: roe deer (Capreolus capreolus) density in the margins of its geographical range. *PLoS One* **9**: e88459.

Versalovic J, Schneider M, De Bruijn F & Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**: 25–40.

Vieira-Pinto M, Morais L, Caleja C, Themudo P, Torres C, Igrejas G, Poeta P & Martins C (2011) Salmonella sp. in game (Sus scrofa and Oryctolagus cuniculus). *Foodborne Pathog Dis* **8**: 739–740.

Webber MA (2002) The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother* **51**: 9–11.

WHO (2011) *Enterohaemorrhagic Escherichia coli (EHEC)*.

World Health Organization (2012) *The evolving threat of antimicrobial resistance*. GPS PUBLISHING, France.

World Health Organization (2014) *Antimicrobial resistance global report on surveillance*.

Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD, Hashsham SA & Tiedje JM (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc Natl Acad Sci U S A* **110**: 3435–3440.

Appendices

Appendix 1. Typical composition of medium (g/litre)

A. 1.1. MacConkey Agar (Merck)

Peptone from gelatin 17.0; peptone from casein 1.5; peptone from meat 1.5; sodium chloride 5.0; lactose 10.0; bile salt mixture 1.5; neutral red 0.03; crystal violet 0.001; agar-agar 13.5.

A. 1.2. ChromoCult ® Coliform Agar (Merck)

Peptone 3.0; sodium chloride 5.0; sodium dihydrogen phosphate 2.2; di-sodium hydrogen phosphate 2.7; sodium pyruvate 1.0; tryptophan 1.0; agar-agar 10.0; Sorbitol 1.0; Tergitol® 7 0.15; 6-chloro-3-indoxyl beta-Dgalactopyranoside 0.2; 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronic acid 0.1; isopropyl-beta Dthiogalactopyranoside 0.1.

A. 1.3. Modified Semi-solid Rappaport Vassiliadis (MSRV; Oxoid)

Tryptose 4.59; casein hydrolysate 4.59; sodium chloride 7.34; potassium dihydrogen phosphate 1.47; magnesium chloride (anhydrous) 10.93; malachite green oxalate 0.037; agar 2.7; pH 5.4 ± 0.2 @ 25°C. Suplement: Novobiocin 10.0 mg.

A. 1.4. *Salmonella Shigella* Agar (*SS* agar; Merck)

Peptones 10.0; lactose 10.0; ox bile 8.5; sodium citrate 10.0; sodium thiosulfate 8.5; ammonium iron(III) citrate 1.0; brilliant green 0.0003; neutral red 0.025; agar-agar 12.0.

A. 1.5. Tryptic Soy Agar (TSA) (Merck)

Peptone from casein 15.0; peptone from soymeal 5.0; sodium chloride 5.0; agar-agar 15.0.

A. 1.6. Buffered Peptone Water (BPW; Merck)

Peptone from casein 10.0; sodium chloride 5.0; disodium hydrogen phosphate dodecahydrate 9.0; potassium dihydrogen phosphate 1.5.

A. 1.7. Xylose-Lysine-Desoxycholate Agar (XLD; Oxoid)

Yeast extract 3.0; L-Lysine HCl 5.0; xylose 3.75; lactose 7.5; sucrose 7.5; sodium desoxycholate 1.0; sodium chloride 5.0; sodium thiosulphate 6.8; ferric ammonium citrate 0.8; phenol red 0.08; agar 12.5; pH 7.4 ± 0.2 @ 25°C.

A. 1.8. Luria-Bertani (LB; Merk)

Tryptone ....10.0 g

Yeast Extract .... 5.0 g

Sodium Chloride .... 10.0 g

A. 1.9. Mueller Hinton Agar (MHA; Merck)

Meat infusion 2.0; casein hydrolysate 17.5; starch 1.5; agar-agar 13.0.

Appendix 2. Samples data

A. 2.1. Wild Boar (*Sus scrofa)*

Twenty-one faecal samples from wild boars were collected in mounts. The data samples are shown in Table 10.

Table 10: List of samples with respective collection date and location.

Sample ID	Date of collection	Place of collection
J1	12-10-2013	Lousã
J2	12-10-2013	Lousã
J3	12-10-2013	Lousã
J4	12-10-2013	Lousã
J5	12-10-2013	Lousã
J6	18-01-2014	Lousã
J7	18-01-2014	Lousã
J8	18-01-2014	Lousã
J9	18-01-2014	Lousã
J10	18-01-2014	Lousã
J11	18-01-2014	Lousã
J12	25-01-2014	Lousã
J13	26-01-2014	Penacova
J14	26-01-2014	Penacova
J15	22-02-2014	Idanha-a-Nova
J16	22-02-2014	Idanha-a-Nova
J17	22-02-2014	Idanha-a-Nova
J18	22-02-2014	Idanha-a-Nova
J19	22-02-2014	Idanha-a-Nova
J20	22-02-2014	Idanha-a-Nova
J21	22-02-2014	Idanha-a-Nova

A. 2.2. Red Deer (*Cervus elaphus)*

Thirty-nine faecal samples from red deer were collected. The data samples are shown in Table 11.

Table 11: List of samples with respective collection date and location.

Sample ID	Date of collection	Place of collection
V1	12-10-2013	Lousã
V2	12-10-2013	Lousã
V3	12-10-2013	Lousã
V4	14-10-2013	Montesinho
V5v	14-10-2013	Montesinho
V6	17-01-2014	Lousã
V7	17-01-2014	Lousã
V8	17-01-2014	Lousã
V9	18-01-2014	Lousã
V10	18-01-2014	Lousã
V11	18-01-2014	Lousã
V12	18-01-2014	Lousã
V13	01-02-2014	Lousã
V15	31-01-2014	Lousã
V16	31-01-2014	Lousã
V17	01-02-2014	Lousã
V18	01-02-2014	Lousã
V19	31-01-2014	Lousã
V20	01-02-2014	Lousã
V21	01-02-2014	Lousã
V22	31-01-2014	Lousã
V23	22-02-2014	Idanha-a-Nova
V24	22-02-2014	Idanha-a-Nova
V25	22-02-2014	Idanha-a-Nova
V26	22-02-2014	Idanha-a-Nova
V27	22-02-2014	Idanha-a-Nova
V28	22-02-2014	Idanha-a-Nova
V30	28-02-2014	Lousã

V31	28-02-2014	Lousã
V32	28-02-2014	Lousã
V33	05-03-2014	Lousã
V34	05-04-2014	Lousã
V35	30-03-2014	Lousã
V36	05-04-2014	Lousã
V37	05-04-2014	Lousã
V39	05-04-2014	Lousã
V40	05-04-2014	Lousã
V41	05-04-2014	Lousã
V42	05-04-2014	Lousã

A. 2.3. Roe Deer (Capreolus capreolus)

Four faecal samples from red deer were collected. The data samples are shown in Table 12.

Table 12: List of samples with respective collection date and location.

Sample ID	Date of collection	Place of collection
V/C-1	14-10-2013	Montesinho
C2-1	18-01-2014	Lousã
C3-1	18-01-2014	Lousã
C4-1	31-01-2014	Lousã